Assessment of the bioactive and nutritional potential of novel food products enriched in *Cystoseira abies-marina* and *Skeletonema* sp. biomass

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II. Abstract

Micro and macroalgae are photosynthetic organisms of large abundance and diversity that contain a variety of compounds with high bioactivity and nutritional value. These compounds have been receiving an increasing interest of researchers due to their potentially positive effect on human health. This has also led to the development of applications for algal biomass, ranging from animal feed to cosmetics, pharmaceuticals, and functional foods. The use of algae biomass in the food industry is still a new concept with the potential to produce healthy foods with added nutritional and bioactive compounds. Although there are many countless studies that support the health benefits of algae, few studies quantify the bioavailability of those nutritional compounds. *In vitro* digestion methods currently being used for studying the bioaccessibility of said nutrients and bioactive compounds, allowing a better understanding of how those compounds can be used. In this work two algae species, microalgae *Skeletonema* sp. and macroalgae *Cystoseira abies-marina,* that are not thoroughly studied were analysed regarding their proximal composition, lipid composition, element composition, phenolic content and relevant bioactivities, as well as their bioaccessibility, in order to understand their potential to be incorporated in food products. Two food products enriched in algae, sauce and cookies, were prepared and analysed, displaying a higher content in polyphenols and higher antioxidant activity than the control in the bioaccessible fraction. The high arsenic concentration found in the macroalgae is also observed in food products prepared with this species requiring a speciation study to assess the risk to human health. The mixture of the food ingredients with *Skeletonema* sp. was beneficial for the bioaccessibility of monounsaturated fats in sauces, which increased with the incorporation of the microalgae. This work reinforced that incorporating algae in food products can be advantageous, thereby paving the way for effective and marketable functional foods.

Keywords: *Cystoseira*, *Skeletonema*, Bioaccessibility, Antioxidant, Lipids

III. Resumo

As algas são organismos fotossintéticos de grande abundância e diversidade que contêm uma interessante variedade de compostos com alta bioatividade e valor nutricional. Esses compostos têm sido alvo de vários trabalhos científicos a fim de averiguar os seus efeitos positivos na saúde humana. A utilização de biomassa de algas para produzir alimentos funcionais é um conceito relativamente novo, mas com enorme potencial para produzir alimentos saudáveis ricos em compostos nutricionais e bioativos. Embora existam muitos estudos que demonstram os benefícios para a saúde das algas, poucos estudos quantificam a biodisponibilidade desses compostos nutricionais e fitoquímicos. Os métodos de digestão *in vitro* são atualmente usados para estudar a bioacessibilidade dos nutrientes e compostos bioativos, permitindo uma melhor utilização desses compostos. Neste trabalho, duas espécies de algas pouco estudadas, a microalga *Skeletonema sp*. e a macroalga *Cystoseira abies-marina*, foram analisadas quanto à sua composição proximal, composição lipídica, composição dos minerais e contaminantes, teor em polifenóis e bioatividades relevantes, bem como quanto à bioacessibilidade desses compostos, de modo a avaliar o seu potencial de incorporação em géneros alimentícios. Dois produtos alimentares distintos com incorporação destas espécies, molho de iogurte e bolachas, foram preparados e analisados, apresentando maior teor em polifenóis e maior atividade antioxidante que o controlo na fração bioacessível. A alta concentração de arsénio verificada na macroalga *Cystoseira abies-marina* refletiu-se nos produtos preparados com esta espécie pelo que seria necessário efetuar um estudo de especiação para avaliar a toxicidade e os perigos para a saúde. Verificou-se que a mistura dos componentes dos produtos com as algas revelou-se benéfica para a bioacessibilidade de ácidos gordos monoinsaturados nos molhos que aumentou no molho com a microalga. Este trabalho reforçou que a incorporação de algas em produtos alimentares é benéfica, abrindo caminho para a comercialização de alimentos funcionais.

Palavras-chave: *Skeletonema, Cystoseira,* Bioacessibilidade, Antioxidante, Lípidos

IV. Resumo Alargado

Algas são um grande e diverso grupo de organismos fotossintéticos, que fazem parte da alimentação humana há milhares de anos em toda a parte do mundo. Podem dividir-se em microalgas, organismos unicelulares, e macroalgas, organismos multicelulares que se dividem, de acordo com o pigmento que contêm, em algas verdes, vermelhas e castanhas. Atualmente, estes organismos têm vindo a ser alvo de atenção por parte da comunidade científica, não só devido às suas propriedades nutricionais, mas também devido à sua variedade de compostos bioativos com efeito positivo na saúde humana. Alguns desses compostos são ácido gordos polinsaturados como o ácido eicosapentaenóico (EPA) ou o pigmento fucoxantina, presente em algas castanhas e algumas diatomáceas, com potenciais efeitos como antioxidantes, anti-inflamatórios e anti carcinogénicos. O alto teor em minerais como iodo, cálcio, magnésio e potássio também torna as algas numa excelente fonte de micronutrientes. Contundo, sendo organismos bioacumuladores, podem acumular metais nocivos presentes na água em quantidades indesejadas como o arsénio, cobre ou chumbo. Hoje em dia as algas são frequentemente estudadas, no entanto estes organismos marinhos são tão diversos e heterogéneos que muitas espécies ainda não são bem conhecidas, em particular há lacunas acerca da sua composição e compostos bioativos.

Embora existam muitos estudos que demonstram a presença de compostos benéficos para a saúde nas algas, poucos quantificam a sua bioacessibilidade/biodisponibilidade. Os métodos de digestão *in vitro* são atualmente usados como uma abordagem para estudar a biodisponibilidade ao aferir a bioacessibilidade dos referidos nutrientes e compostos bioativos, permitindo uma melhor compreensão de como esses compostos podem ser usados. Portanto, o potencial para utilizar algas como ingrediente para incorporar num género alimentício e acrescentar-lhes propriedades benéficas é imenso e tem vindo a ganhar relevância. Como este conceito é relativamente recente, ainda não há muita informação sobre como as propriedades das algas afetam o alimento nem que compostos bioativos ficam de facto biodisponíveis a ser absorvidos pelo organismo humano.

Neste trabalho duas espécies de algas pouco estudadas, a microalga diatomácea *Skeletonema* sp. e a macroalga castanha *Cystoseira abies-marina,* foram analisadas quanto à sua composição proximal, perfil de ácidos gordos, composição mineral, conteúdo polifenólico e atividade antioxidante (medida por DPPH, FRAP e ABTS), a fim de compreender seu potencial de serem incorporados em géneros alimentícios. O foco deste trabalho foi desenvolver dois géneros alimentícios distintos (molho de iogurte e bolachas) que incorporassem a microalga *Skeletonema* sp. e a macroalga *Cystoseira abies-marina* e estudar de que modo as algas afetam as propriedades nutricionais e bioativas destes produtos bem como a sua bioacessibilidade.

Em relação às propriedades das algas, verificou-se que o teor de lípidos e proteína da microalga foi superior, mas o teor em hidratos de carbono foi inferior ao da alga *C. abies-marina*. Como o teor lipídico da macroalga *C. abies*-marina foi muito reduzido (0,79 ± 0,13 %) o ensaio da bioacessibilidade dos lípidos foi apenas realizado na diatomácea e, consequentemente, apenas nos produtos alimentares com *Skeletonema*. No entanto, os lípidos totais da *Skeletonema* acabaram por não ser bioacessíveis. O perfil de ácidos gordos de ambas as espécies foi caracterizado pela abundância em ácidos gordos saturados e pelo conteúdo relevante de ácidos gordos polinsaturados (PUFA). *C. abies-marina* apresentou maior quantidade de PUFA, mas *Skeletonema* sp. apresentou uma maior razão ω3/ω6, com uma percentagem de EPA de 4,20 ± 0,29 %. A distribuição das classes lipídicas foi estudada com recurso a cromatografia de camada fina (TLC) na microalga *Skeletonema,* verificando-se, a ausência de triacilgliceróis (TAG) e a predominância de ácidos gordos livres (FFA). O conteúdo polifenólico total foi alto nas duas espécies, mas a macroalga obteve um valor superior e muito considerável de 8,43 ± 0,73 mg GAE/g de peso seco. O efeito antioxidante de extratos aquosos das duas espécies revelou ser considerável. A microalga apresentou maior atividade antioxidante medida por DPPH, mas menor capacidade antioxidante total medida por FRAP do que a *C. abies-marina*. Os resultados do ABTS não mostraram diferenças entre as duas espécies, ambas com altos valores de 41,9 ± 0,2 µmol de equivalentes Trolox por g de peso seco para a microalga e 43,5 ± 1,2 µmol de equivalentes Trolox por g de peso seco para a macroalga. Devido às restrições de material e tempo, o modelo de digestão *in vitro* dirigido para os bioativos foi aplicado apenas na espécie *Cystoseira abies-marina*, uma vez que apresentou resultados mais promissores em relação a estes compostos. Assim sendo, a atividade antioxidante e o teor de polifenóis na fração bioacessível foram determinados apenas nos alimentos com a macroalga. A composição mineral das duas espécies desvendou algumas diferenças e semelhanças entre as espécies. Cálcio e sódio foram os minerais mais abundantes em *Skeletonema* sp. e potássio e sódio foram os mais abundantes na macroalga. No que diz respeito aos contaminantes, cobre e chumbo encontravam-se em concentrações semelhantes e baixas nas duas espécies. No entanto, o arsénio apresentou concentrações consideráveis na macroalga *C. abies-marina.* O modelo de digestão *in vitro* dos minerais foi também realizado apenas na espécie *C. abies-marina*, e consequentemente apenas nos produtos alimentares com esta alga. Os resultados revelaram que o arsénio, potássio e magnésio foram os mais bioacessíveis em contraste com o cobre, zinco e fósforo.

Os produtos alimentares foram preparados com 2 % de biomassa de alga no caso do molho de iogurte e 3 % de biomassa de alga no caso das bolachas. A composição proximal das três bolachas e três molhos não revelou diferenças entre si, sendo necessário utilizar uma maior quantidade de alga, no entanto o aspeto sensorial dos produtos seria fortemente

afetado. A percentagem de bioacessibilidade de lípidos totais foi alta com resultados entre 53-90 %. O perfil de ácidos gordos obtidos por catálise ácida revelou que nas bolachas os ácidos gordos polinsaturados (PUFA) são os mais abundantes, com destaque para o ácido linoleico (18:2 ω6) e o ácido alfa-linolénico, um ácido gordo ómega 3 essencial que também apresentou percentagens relevantes. O ácido oleico (18:1) foi o segundo ácido gordo mais abundante. O ácido palmítico (16:0) foi o SFA maioritário encontrado nas bolachas. Nos molhos os ácidos gordos monoinsaturados (MUFA) foram os maioritários com percentagens totais cerca de 70 %. O ácido oleico foi o ácido gordo mais abundante. Em relação aos SFA, o ácido palmítico foi de novo o mais relevante. PUFA apresentaram uma baixa percentagem, com destaque para o ácido linoleico. O perfil de ácidos gordos das duas bolachas e dos dois molhos apenas apresentaram diferenças no teor em ácido mirístico (14:0) e no ácido palmitoleico (16:1) com os produtos com microalga a apresentar maiores teores. Após a digestão, verificou-se que os MUFA foram os ácidos gordos mais bioacessíveis em oposição aos SFA que apresentaram baixa bioacessibilidade. Os ácidos gordos da bolacha com microalga foram menos bioacessíveis que os do controlo, já nos dois molhos verificou-se que os MUFA foram mais bioacessíveis com a adição da microalga. A distribuição das classes lipídicas nos produtos revelou uma clara redução de triacilgliceróis (TAG) na fração bioacessível acompanhada de um aumento de FFA, MAG e DAG. No caso das bolachas os TAG não desapareceram completamente revelando que a digestão foi incompleta, já nos molhos a digestão foi completa. Relativamente aos compostos bioativos e bioatividades, o molho com *Skeletonema* sp. e a bolacha com *C. abies-marina* apresentaram um maior teor em polifenóis que os restantes produtos. Os produtos com a macroalga apresentaram uma maior atividade antioxidante medida por DPPH e FRAP, sendo que neste último método os molhos apresentaram maior atividade. A atividade antioxidante medida por ABTS revelou que a bolacha com macroalga e o molho com microalga obtiveram os melhores resultados, com os molhos mais uma vez a ter maior atividade antioxidante que as bolachas. Estas diferenças podem estar associadas ao facto de as bolachas terem sofrido um tratamento térmico que afetou os compostos responsáveis pela atividade antioxidante. Na fração bioacessível, o teor total em polifenóis e o ensaio de FRAP revelaram grandes percentagens de bioacessibilidade, contrastando com os baixos fatores de bioacessibilidade do ensaio DPPH. Relativamente à composição mineral, o sódio foi o elemento mais abundante nos dois produtos. Os molhos apresentaram uma maior quantidade de zinco, potássio, cálcio, sódio e fósforo. Devido à elevada concentração de arsénio encontrada na macroalga, os produtos com a adição dessa espécie revelaram maiores teores que o respetivo controlo. Os dois produtos apresentaram percentagens semelhantes e altas de bioacessibilidade de sódio, magnésio, cálcio, arsénio e fósforo. Zinco e chumbo foram os minerais menos bioacessíveis.

Para concluir, sem dúvida que as algas podem ser utilizadas com um ingrediente que enriquece nutricionalmente um género alimentício. Neste estudo, os produtos com algas obtiveram uma melhoria significativa na sua atividade antioxidante e conteúdo em polifenóis após a digestão. O elevado teor em arsénio encontrado na macroalga requer estudos adicionais de especiação de arsénio de modo a avaliar o nível de toxicidade e os potenciais riscos associados ao consumo. Seria também interessante efetuar um prova de análise sensorial dos produtos, tendo em conta que o sabor é uma das características mais importantes de um produto alimentar.

V. Table of Contents

VI. List of Figures

VII. List of Tables

VIII. List of Abbreviations

AA – Araquidonic Acid

ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

ALA - α-linolenic acid

CH – Cholesterol

DAG – Diacylglycerol

DHA – Docosahexaenoic acid (22:6 ω3)

DPPH - 2,2-diphenyl-1-picrylhydrazyl

EPA - Eicosapentaenoic acid (20:5 ω3)

FA – Fatty acid

FAME - Fatty acid methyl esters

FFA – Free fatty acids

FRAP - Ferric Ion Reducing Antioxidant Power

GA – Gallic acid

GAE – Gallic acid equivalents

IPMA – Instituto Português do Mar e da Atmosfera

MAG – Monoacylglycerol

MUFA – Monounsaturated fatty acid

PBS - Phosphate buffered saline

PUFA – Polyunsaturated fatty acid

SDA - Stearidonic acid

SFA – Saturated fatty acid

TAG – Triacylglycerol

TLC – Thin layer chromatography

TPTZ - 2,4,6-tripyridyl-s-triazine

1. Introduction

1.1 Food and health in the XXIst century

The last decade has been characterized by important transformations in the food consumers preferences and concerns. This has affected mostly Western countries and Portugal has been no exception. Indeed, there has been a significant change in eating habits and food expectations, with consumers increasingly considering food and health as two inseparable aspects. In addition, the EU, through the White Paper entitled 'Together for Health: A Strategic Approach for the EU', has signaled that it is essential to match existing foods on the market with legal requirements with full inclusion of nutrition and health and wellbeing. These changes inevitably have repercussions for all food products and represent a challenge that can only be met with deeper knowledge and new concepts, thereby opening up new opportunities arising from undervalued and underutilized raw materials.

The food sector faces an existential challenge arising from the intersection of two contradictory currents. On the one hand, there is an increasing trend to value the sensory quality of foods, being some traditional foods very positively appreciated and, at the same time, exposed to a strong pressure to recreate/update themselves. On the other hand, the growing health awareness by the average consumer often poses a dilemma because it either entails a loss in the product's sensory quality as a result of less savoury alternative healthy ingredients or it only marginally improves health benefits (or reduces risks) without changing any fundamental in the product. The pressures are further exacerbated by consumer demands for foods whose ingredients are produced without compromising the environment and in a thoroughly sustainable way. The application of underutilized and abundant natural resources is part of the response to this challenge.

1.2 Algae as a food source

Algae have been part of the human diet for thousands of years in all parts of the world (Borowitzka, 1998), being used as an important dietary component in countries like China, Japan and Korea. Microalgae, mainly the blue-green algae or cyanobacteria, such as *Arthrospira platensis*, also known as *Spirulina*, have been used as food source in Africa and South America for centuries (Ciferri, 1983).

Algae's high concentration of vitamins and minerals represents an obvious health benefit. Algae contain more vitamin A, B-12, and C, riboflavin, and niacin than fruits and vegetables from regular land cultivars (García-Casal et al., 2007). During the past decades, microalgae biomass has been used almost exclusively in the health food market. Over 75 % of the annual

biomass production has been solely dedicated to the manufacture of powders, tablets, and capsules of microalgae production (Chacón-Lee and González-Mariño, 2010). Nowadays, seaweeds are very attractive to consumers and the food industry due to their low calorie content and high content of key nutrients such as carbohydrates, vitamins, minerals and dietary fibre (Cofrades et al., 2017).

All of this algae biomass could be incorporated into food products, producing healthy foods rich in substances with important health benefits due to antioxidant and anti-inflammatory effects, allowing this industry to diversify and grow in ways previously unrealized. However, some concerns over algae consumption have been raised, mainly over toxicity, ingestion of contaminants, heavy metals, and intake of high levels of iodine (Bouga and Combet, 2015), requiring further studies to address these concerns.

1.3 Algae: an immense diversity

Algae are an incredibly large and diverse group of marine organisms, and are considered to be the most efficient biological system for harvesting solar energy and for the production of organic compounds via the photosynthetic process (Shalaby, 2011). These photosynthetic organisms are among the most heterogeneous forms of life, considered the true survivors of the planet by being able to survive the most critical climate conditions and occupy virtually all niches on earth (Mendis and Kim, 2011). Algae can be divided in microalgae, microscopic single cells that may be prokaryotic or eukaryotic, and macroalgae, multicellular, large-size algae, visible with the naked eye.

Macroalgae, most commonly referred as seaweed, provide oxygen and/or organic compounds to the majority of marine living organisms, acting as a primary producer in the aquatic food chain. They can be divided, according to the presence of a specific pigment, in *Chlorophyceae* (green), *Phaeophyceae* (brown) and *Rhodophyceae* (red) (Chapman and Chapman, 1980). *Chlorophyceae* are green seaweed due to the presence of chlorophyll in their chloroplasts, with their colour depending on interactions between chlorophylls and other pigments, such as xanthophylls and β-carotene (Afonso et al., 2018). The pigment responsible for the brown colour of the *Phaeophyta* species is fucoxanthin, the red colour of the *Rhodophyta* species comes from phycobilins (Øverland et al., 2019). The presence of these different phytopigments in algae is related to their sea habitat. Thus, green macroalgae, which are able to absorb large amounts of light energy, abound in coastal waters, while red and brown macroalgae prevail at greater depths, where penetration of sunlight is limited (Bocanegra et al., 2009).

Macroalgae have shown to provide a rich source of natural bioactive compounds with antiviral, antifungal, antibacterial, antioxidant, anti-inflammatory, hypercholesterolemia and

hypolipidemic and antineoplasteic properties (Shalaby, 2011). They also have been described as a good source of iodine, which is important in metabolic regulation and growth, although consumption of very large amounts may induce some negative toxic effects (Taboada et al., 2010). Seaweeds possess useful technological properties, largely associated with the dietary fibre they contain. They provide hydrocolloids (agar-agar, alginates used as stabilizers, thickeners and fillers), pigments and vitamins that are widely used in food and pharmaceutical industry.

Microalgae are a very heterogeneous group of organisms, with a wide diversity of physiological and biochemical characteristics. There are various groups of microalgae, with the most abundant being the eukaryotic diatoms (*Bacillariophyceae*), green algae (*Chlorophyceae*) and golden algae (*Chrysophyceae*) (Madeira et al., 2017). The cyanobacteria (*Cyanophyceae* or blue-green algae) are also an important group which are prokaryotic organisms. Currently, the most used species in research and production are: *Arthrospira* (formerly known as Spirulina, blue-green algae), *Chlorella*, *Dunaliella* and *Haematococcus* (Madeira et al., 2017). These marine organisms can serve as a reliable source of natural products since the quality of the microalgal cells can be controlled, so that they contain no toxic substances, by using clean nutrient media for growing the microalgae (Shalaby, 2011). Microalgae possess an interesting nutritional value with many vitamins and minerals, such as vitamin A, C, B_1, B_2, B_6, niacin, iodine, potassium, iron, magnesium and calcium (Priyadarshani and Rath, 2012). *Arthrospira,* being an extremely nutritious food, has gained worldwide popularity as a food supplement. It has been shown to be an excellent source of proteins, lipids, mainly PUFA's (polyunsaturated fatty acids), pigments, vitamins and phenolics (Colla et al., 2007).

There are still many algae species whose composition and properties are virtually unknown. Even among algae of the same species and strain, simple factors such as their geographical location can affect their composition and properties. This work was focused on two different species of algae, the diatom *Skeletonema* sp. and the brown seaweed *Cystoseira abies-marina.*

1.3.1 Properties and applications of diatoms

Diatoms are important primary producers, estimated to be responsible for almost 25% of global carbon fixation (Mishra et al., 2017). These organisms are among the most productive and environmentally flexible eukaryotic microalgae on the planet (Hildebrand et al., 2012). Diatoms are highly robust organisms, and can inhabit virtually all photic zones from the equator to seemingly inhospitable sea ice (Jamali et al., 2012). These phytoplanktons are categorized into centric diatoms (*Coscinodiscophyceae*) and pennate diatoms (*Fragilariophyceae*; no raphe and *Bacillariophyceae*; with raphe), existing either as unicellular or colonies, filaments,

ribbons (*Fragilaria*), fans (*Meridion*), zigzags (*Tabellaria*), or stellate (*Asterionella*) (Mishra et al., 2017).

Diatoms abundance and wide distribution make them ideal tools for a wide range of applications. Due to ease of mass cultivation and high lipid productivity, diatoms have been studied for their potential for biofuel production (Maeda et al., 2017). These organisms have also found important applications health foods and as bioremediators of contaminated water (Bozarth et al., 2009). Diatoms are also widely used as the source of compounds with diverse structure forms and biological activities (Lenin et al., 2015). Various studies have shown that some diatoms are rich in bioactive compounds such as fucoxanthin, with antioxidant, anti-inflammatory, anticancer, anti-obese, antidiabetic, antiangiogenic, and antimalarial activities (Lauritano et al., 2016).

The most nutritionally relevant biomolecules produced by diatoms are polyunsaturated fatty acids like eicosapentaenoic acid (EPA) arachidonic acid, docosahexaenoic acid (DHA) and other omega-3 fatty acids (Bozarth et al., 2009). EPA and DHA are considered essential fatty acid, since humans cannot synthetize them. They reduce cardiovascular disease and are precursors of important tissue hormones, being present in 20% of the brain's grey matter (Kroth, 2007).

1.3.2 Characteristics of brown seaweeds

Brown algae are the largest seaweeds, up to 35–45 m in length for some species and extremely variable in shape, being an extensively studied group (Makkar et al., 2015). Brown algae are of lesser nutritional value than red and green algae, due to their lower protein content and higher mineral content; however brown algae contain a number of bioactive compounds (Makkar et al., 2015). Brown seaweed contain complex carbohydrates and polysaccharides mainly alginates, sulphated fucoidans and laminarin (Øverland et al., 2019). These seaweeds can also contain sugar alcohols such as mannitol that can be up to 25% of their dry weight (Holdt and Kraan, 2011).

Average total lipid content of brown seaweed has been reported to be 1-6 % per dry weight, containing high level of omega-3 polyunsaturated fatty acids (PUFA) such as 18:4 ω3 (SDA) and 20:5 w3 (EPA) together with omega-6 PUFA, 20:4 ω6 (arachidonic acid, AA) (Airanthi et al., 2011). Brown seaweed also contain phlorotannins, phenolic compounds that exhibit wide range of biological activities: anti-diabetic activity, antiproliferative activity, acetylcholinesterase inhibition activity, anti-HIV activity, and many others (Cikoš et al., 2018).

Brown macroalgae within the genus *Cystoseira* are some of the most relevant species found throughout the Mediterranean and the adjacent Atlantic coasts (Valdazo et al., 2017), however there is still little data available for some species. Brown algae of the genus

Cystoseira are known to contain enzyme inhibitors, cell division inhibitors, antibacterial and antitumor constituents (Abourriche et al., 1999).

1.3.3 Description and characteristics of the diatom *Skeletonema* sp. and brown seaweed *Cystoseira abies-marina*

Skeletonema species are common phytoplankters, especially in coastal estuarine and marine environments where they often form dense blooms (Kooistra et al., 2008). These organisms can tolerate a wide variety of light regimes and temperatures, and are able to grow readily in various media (Kumar and Prabu, 2015). It is reported that extracts of *Skeletonema costatum* are capable of scavenging a wide range of synthetic and naturally occurring free radicals and could be utilized as a good natural source of antioxidants and possible food supplement (Lenin et al., 2015). This species also contains essential fatty acids ω3 and ω6, vitamins A, B1, B2, B3, B5, B6, B12, C, D and other valuable nutritional components (Kumar and Prabu, 2015). Additionally, the species *Skeletonema marinoi* produce bioactive polyunsaturated aldehydes that have shown anticancer effects in lung cancer A549 and colon COLO 205 tumour cells (Sansone et al., 2014).

Cystoseira abies-marina (S.G. Gmelin) C. Agardh is a brown edible macroalgae (Phaeophyta) which belongs to Cystoseira genus and is distributed in the Mediterranean, Macaronesian Region and in the coast of Africa (Guiry, 2019). *C. abies-marina* is a caespitose seaweed with large numbers of erect branches, up to 50 cm long (Valdazo et al., 2017). This species is abundant in Azores Region, being traditionally used as a fertilizer and food seasoning. *Cystoseira abies-marina* is a species identified as possessing some interesting compounds, such as meroterpenoids and fucoxanthin, and is also a good potential source of phenolic compounds, including phlorotannins (Montero et al., 2014). Barreto *et al.*(2012) evaluated the pharmacological potential of this seaweed and demonstrated a high and selective antiproliferative activity against HeLa cells. Although there is still little information regarding antioxidant activity in macroalgae, the *Cystoseira* genus generally has one of the highest total phenolic levels and antioxidante activities among *Phaeophyceae* macroalgae, such as *Fucus serratus*, *Dictyota dichotoma*, *Bifurcaria bifurcata*, *Sargassum horneri* and *Alaria crassifolia* (Zubia et al., 2009).

Although these two species are very different and are scarcely studied, they both are known to have relevant bioactivities having potential to be incorporated in food products and acting as a functional ingredient.

1.4 Potential uses of algal biomass

The potential uses for microalgae as new sources of valuable chemicals and other products recently have regained wide interest. The use of marine microalgae for applications, as such or as extracts, in areas so diverse as human nutrition and feed in aquaculture, as biofertilizers and in treatment of effluents, as anti-inflammatory, antiallergic and analgesic agents and its biomass being used for the production of biofuel (Khan et al., 2018). The average protein quality of most of the algae examined is equal, sometimes even superior, to that of conventional plant proteins (Chacón-Lee and González-Mariño, 2010), having potential to be used as protein supplements. There are also reports of algae having historical use as food, medicine, fertiliser and animal fodder (Kenicer et al., 2000). Nowadays, microalgae can produce in bioreactors, in a large scale, high commercial value chemicals, including carotenoids (Borowitzka, 2010) polyunsaturated fatty acids, like DHA (Mendes et al., 2009), and phycobilins (Singh et al., 2005). The content of lipids, proteins (amino acids), carbohydrates and vitamins of microalgae species is the main reason to why these organisms as feed source for aquaculture animal (Kumar and Prabu, 2015).

Macro- and microalgae extracts are already used as sources of cosmetic products with biologically active ingredients purporting to have medical or drug-like benefits, like phycobilin pigments, algae-derived carotenoids (Borowitzka, 2013). Seaweeds are also a great source of iodine, for that reason has many uses in medicine to treat iodine deficiency (Makkar et al., 2015).

1.5 Nutritional quality assessment and the importance of bioaccessibility

For good health, it is necessary to have a balanced diet that is rich in minerals, vitamins and dietary fiber. For this reason, algae have been recommended as dietary supplements because of their composition. Although, algae have been consumed in Asia for a long time, the current interest in these marine organisms has led western countries to develop algae industries. These industries require data on the nutritional composition, as well as the total concentration of essential and contaminants elements, before the commercialization of the products. However, the total concentrations may not always reflect the available amounts of the nutrients and contaminants ingested, making necessary to know the bioavailable amount to humans (Afonso et al., 2015). Many studies report the potential nutritional or bioactive content of different algae, but much fewer studies quantify the bioavailability of nutrients and phytochemicals from algal foods.

Bioavailability is difficult to assess as it varies with different foods and food components and with gastrointestinal conditions, depending on several processes such as digestion,

absorption, transport, utilization and elimination (Domínguez-gonzález et al., 2010). According to Versantvoort *et al*. (2005), the oral bioavailability of any element or compound is the result of three processes: a) bioaccessibility, b) the degree of transport across the intestinal epithelium and c) degradation of the compound in the liver and intestine. In order to determine the bioavailability of a compound, evaluating its bioaccessibility is a useful approach as it can be considered as an indicator of the maximal oral bioavailability. Bioaccessibility can be described by the amount of a compound that is released from the matrix and is solubilized into the water phase, becoming available for absorption through the gut wall (Lucas-González et al., 2018), but can be significantly different depending on the food source, food processing or culinary treatment (Roselli et al., 2017; Versantvoort et al., 2005)

1.6 *In vitro* digestive model as a tool to assess bioaccessibility

Food digestion is a very complex process that requires the involvement of mechanic and enzymatic transformations, allowing the release of nutrients and phytochemicals from the food matrix to be latter absorbed by the organism The knowledge of the transformations that occur in the digestive process by assessing the bioaccessibility of a nutrient or contaminant in algae matrix, would be of great help for designing functional foods, since the processing conditions that maximize the health benefits of bioactive compounds would be evaluated.

In vitro digestion methods are currently being used as an approach to study several aspects related to foods biotransformation within the gastrointestinal tract, reproducing the digestion process in the laboratory, under controlled, accurate, and reproducible conditions (Calvo-Lerma et al., 2018). *In vitro* digestion assays simulate the physiological conditions, like chemical and enzymatic composition of the digestive fluids, pH and typical residence time in each digestive step of digestion *in vivo*, and are useful tools for studying and understanding changes, interactions, as well as the bioaccessibility of nutrients and bioactive compounds (Lucas-González et al., 2018; Versantvoort et al., 2004).

Regarding these *in vitro* models, it should be stressed that most studies only address the availability for intestinal absorption. For this purpose, there is a division between static and dynamic digestive modelling (Cardoso et al., 2015). In the static methodologies, the biochemical reactivity found in the human gastrointestinal tract (oral cavity, gastric environment, and intestinal lumen) is sequentially simulated. Some agitation is applied to simulate the peristaltic movements, but only as a condition alongside temperature and pH for the reactions progress (Kabak and Ozbey, 2012; Versantvoort et al., 2004). Dynamic methodologies are intended to be more realistic, encompassing various phenomena that occur *in vivo*, such as, shear, mixing, hydration, or peristalsis. Moreover, these methodologies attempt to simulate how conditions change over time during each main digestive stage. Namely, concentration of

the enzymes, pH, viscosity, particle size, and nutrient partitioning are not constant in each of these stages (Wittsiepe et al., 2001).

The choice of a static or a dynamic digestion system is worthy of further study. A recent work compared a static methodology with a dynamic model for toxic elements (Torres-Escribano et al., 2011). These authors found out that there are significant differences between the intestinal availability of metals for uptake obtained by the two models. It was not possible to reach a final verdict pointing to a superiority of dynamic over static models.

1.7 Conception and development of innovative algal functional foods

Nowadays there is an increasing consumer interest with regard to products that can promote health, presenting a challenge to the food industry. Hence, food producers have been focusing in the development of "functional foods" or "nutraceuticals". These terms have no legal status in many nations, but describe foods that contain bioactive compounds, or phytochemicals, that may benefit health beyond the role of basic nutrition, in a way that is relevant to either an improved state of health and well-being and/or a reduction of the risk of disease (Borowitzka, 2013; Wells et al., 2017). Being widely available, seaweeds and microalgae are attracting attention to the benefits associated with its consumption, having potential to act as a functional food and ingredient (Bouga and Combet, 2015; Cofrades et al., 2017; Mendis and Kim, 2011).

There are a few studies that use algae as a functional ingredient. Gouveia et al. (2008) produced biscuits enriched with ω-3 fatty acids by the inclusion of *Isochrysis galbana* biomass. Fradique et al. (2010) incorporated *Chlorella vulgaris* and *Spirulina maxima* biomass in pasta products, thereby increasing their nutritional value. Cofrades et al. (2017) studied a formulation of seaweed-enriched meat with health-promoting compositions in terms of: less Na, fat and saturated fatty acids, and promotion of enhanced intake of dietary fibre, polyphenols, minerals and ω3 PUFA's. In general, the developed products met the objectives and healthier foods with good potential in the functional food market were attained.

The path from algal research to the launching of new food products or dietary supplements is strongly affected by industrial, regulatory, and nutritional considerations, which vary from country to country and with the trend being to greater regulation (Wells et al., 2017). Moreover, the commercial success of high-value products from microalgae is not guaranteed and relies, of course, on the availability of a market and the ability to produce the algal product at a competitive price. One of the critical algae characteristics is taste. Although most are edible, whether they are palatable to the general population is an important factor to consider.

Accordingly, the conception and development of algal functional foods raises several problems and interweaves multiple dimensions that range from the technological viability and

economic feasibility to the regulatory issues and, necessarily, to the sensory acceptability and associated health benefits.

2. Objectives

Seaweeds and microalgae have been receiving a growing attention by researchers in recent years. However, these marine organisms are so diverse and heterogeneous that many species lack information regarding their composition and bioactive compounds. Furthermore, using these organisms as an ingredient to produce a “functional food” is a relatively new concept, meaning that there is little information about how algae properties affect a food product and how much of their bioactive compounds are bioaccessible.

The main goal of this work was to develop two distinct food products (cookie and yogurt sauce) using the microalgae *Skeletonema* sp. and the macroalgae *Cystoseira abies-marina* and to study if its nutritional and bioactive properties increase with the incorporation of the algae biomass and to determine if these properties are bioaccessible. The first phase of this study was to analyse the composition, lipid and fatty acid profile, total phenolic content and antioxidant activity (measured by DPPH, FRAP and ABTS) of the species used. The information regarding the seaweed *Cystoseira* was provided by IPMA. The second phase was to select adequate food concepts and prepare food products that could maximize the algae properties, without disregard of the sensory aspect of the food. The third phase was the study of the composition of both products with the different algal biomass. Finally, the fourth phase consisted in the application of the *in vitro* digestive model and analysis of the bioaccessible fraction of the products.

3. Materials and Methods

3.1 Sample Collection and Preparation

3.1.1 Algae species

The species used in this study were the brown seaweed, *Cystoseira abies-marina*, harvested in the mid-north Atlantic island Faial, belonging to the Portuguese arquipelago Azores. The seaweed was supplied by the company seaExpert, sun dried and packed in black plastic bags to the Portuguese Institute of the Sea and Atmosphere (IPMA) laboratory in Lisbon. An adequate amount of *Cystoseira* was then freeze dried, minced and stored at – 80 ºC. The microalgae *Skeletonema* sp. was provided by Necton S.A. (Necton, Companhia Portuguesa de Culturas Marinhas, S.A., Olhão, Portugal). The biomass was grown in tubular photobioreactors using a semi-continuous cultivation approach and cultures were harvested by centrifugation. The biomass was freeze-dried before being sent to IPMA laboratory, where it was kept at – 80 ºC until further analysis.

Figure 1 - Biomass of freeze dried *Skeletonema* sp. (Inês Guarda, 2019).

Figure 2 - Sun dried seaweed *C. abies-marina* (Inês Guarda, 2019).

3.1.2 Food products

The food products chosen to be prepared were yogurt sauce and cookies for they are of easy preparation and enable a straight forward incorporation of the algal biomass. Also, the two food products require different preparation conditions, since cookies undergo high temperatures. Seaweed and microalgae affect greatly the sensory properties of the food products, hence used quantities were low. Moreover, other similar products with incorporation of algal biomass were taken as reference. Accordingly, it was added 2 % of the total mass of the ingredients to the sauce and 3 % of the total mass of the ingredients to the cookies. The algal biomass incorporated in the food products was freeze dried.

The ingredients and the recipe of the yogurt sauces and cookies are presented in Annex **7.1** and **7.2**. The prepared sauces and cookies are illustrated in **Figure 3** and **Figure 4**, respectively. An adequate amount of the products was freeze dried and kept at -80 ºC.

Figure 3 - Yogurt sauces prepared for this study, control (CTR), with 2 % of *Skeletonema* sp. (SKT) and 2 % of *Cystoseira abies-marina* (CYS) (Inês Guarda, 2019).

Figure 4 - Cookies prepared for this study, control (CTR), with 3 % of *Skeletonema* sp. (SKT) and 3 % of *Cystoseira abies-marina* (CYS) (Inês Guarda, 2019).

3.1.3 Nutrition label of the food products

Food labels provide full information of the content and composition of products, so that consumers can make their choices, thus controlling their health and satisfy their interests. As of December 13, 2016 EU Regulation No 1169/2011 on the provision of food information to consumers, makes nutrition labelling mandatory whether or not food products have health claims (EU, 2011). The nutrition labels of the food products prepared were calculated according to EU Regulation No 1169/2011 using the food composition database provided by Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA, 2019) are presented in **Table 1**.

Table 1 - Nutrition values calculated for the prepared food products. These values correspond to the control products, thus the algae was not considered for the calculation.

	Per 100 g of Sauce	Per 100 g of Cookie
Energy (kJ/kcal)	267/64	1808/432
Fat (g)	3.8	22
of which saturates (g)	0.7	4.7
Carbohydrate (g)	4.5	53.9
of which sugars (g)	3.9	19.4
Fibre (g)	0.5	1.5
Protein (g)	2.6	4
Salt (g)	0.3	0.3

3.2 *In vitro* Digestion Model

To determine the bioaccessible total lipids, fatty acid content, antioxidant activity and essential elements, it was used an *in vitro* digestion model. The digestion model implemented was developed by Versantvoort et al. (2004) and modified by Afonso et al. (2015), which includes three steps that simulate the digestive human processes in the mouth, stomach, and small intestine. The composition of digestive juices (saliva, gastric, duodenal and bile) were prepared accordingly. The chemicals KCl, NaH_2PO_4, Na_2SO_4, NaCl, $NaHCO_3$, HCl, $CaCl_2.2H_2O$, KH_2PO_4 and $MgCl_2$ were obtained from Merck (Darmstadt, Germany). NH_4Cl was obtained from Fluka (Buchs, Switzerland) and all other chemicals were obtained from Sigma (St. Louis, MO, USA). For duodenal juice, trypsin and α-chymotrypsin from Sigma (St. Louis, MO, USA) were also added. The quantities of these two enzymes were estimated on the basis of the work by Gatellier and Santé-Lhoutellier (2009).

Initially, it was weighed 1.5 g of sample (0.5 g if sample was freeze-dried) and added 4 mL of the artificial saliva to the matrix at a pH 6.8 ± 0.2 for 5 min, then 8 mL of artificial gastric juice (pH 1.3 ± 0.02 at 37 ± 2 ºC) was added, with an adjust of pH to 2.0 ± 0.1. Subsequently, the samples were placed in a head-over-heels movement (37 rpm at 37 ± 2 ºC) for 2 hours to simulate the digestion conditions. After cooling, a mixture of 8 mL of artificial duodenal juice (pH 8.1 ± 0.2 at 37 ± 2 ºC), 4 mL of bile (pH 8.2 ± 0.2 at 37 ± 2 ºC), and 1.33 mL of HCO_3 solution (1 M) was added simultaneously. The final pH of the mixture was set at pH 6.5 ± 0.5 and then agitated for a further 2 hour period in a head-over-heels movement (37 rpm at 37 ± 2 ºC). The obtained solution after simulated digestion was centrifuged at about 2750 × g, during 5 min, in order to separate the non-digested from the bioaccessible fraction. The antioxidants content, total lipids and fatty acid content were then analysed in the bioaccessible fraction, and the essential elements were analysed in both fractions (bioaccessible and non-digested).

To estimate the percentage of a constituent (C) in the bioaccessible fraction the following formula was used:

$$\%\ \mathrm{C\ bioaccessible} = \frac{\mathrm{mC\ bioaccessible}}{\mathrm{mC\ initial}} \times 100 \quad \textbf{(1)}$$

Where mC is the mass of the constituent (antioxidant, lipids or fatty acids) in the initial and bioaccessible fraction of the product or algae.

To determine the percentage of an element (E) in the bioaccessible fraction the following formulas were used:

$$\%\ \mathrm{E\ bioaccessible} = \frac{\mathrm{mE\ bioaccessible}}{\mathrm{mE\ bioaccessible} + \mathrm{mE\ non-digested}} \times 100 \quad \textbf{(2)}$$

$$\%\ \mathrm{E\ non-digested} = \frac{\mathrm{mE\ non-digested}}{\mathrm{mE\ bioaccessible} + \mathrm{mE\ non-digested}} \times 100 \quad \textbf{(3)}$$

Where mE is the mass of the element in the non-digested and bioaccessible fraction of the product or algae.

3.3 Proximate Composition

3.3.1 Moisture and ash

The moisture and ash content were determined in initial samples using the AOAC Methods (AOAC, 2000). This analysis calculates the amount of water and the inorganic material present in the samples, respectively. To calculate the moisture content it was used the following formula:

$$\%\ \mathrm{Moisture}\ \left(\%\ \frac{\mathrm{m}}{\mathrm{m}}\right) = \frac{(\mathrm{m1} - \mathrm{m2})}{(\mathrm{m1} - \mathrm{m3})} \times 100 \quad \textbf{(4)}$$

Where m1 corresponds to the mass of the crucible containing the moist sample (g), m2 to the mass of the crucible with the dry sample (g) and m3 the crucible mass (g).

To determine the ash content, the crucibles containing the dried sample were placed in the muffle furnace at 550 ºC. After 24 hours the crucibles were removed, cooled for 30 minutes and then weighed. The crucibles were placed in the furnace again at the same temperature for 30 minutes, removed, cooled and weighed. The process was repeated until the weight stopped decreasing or until the difference between consecutive weighings was less than 1 mg. To calculate the ash content (on dry weight basis), the following formula was used:

$$\%\ \mathrm{Ash}\ \left(\%\ \frac{\mathrm{m}}{\mathrm{m}}\right) = \frac{(\mathrm{m1} - \mathrm{m2})}{(\mathrm{m3} - \mathrm{m2})} \times 100 \quad \textbf{(5)}$$

Where m1 corresponds to the mass of the crucible containing the ash (g), m2 to the mass of the crucible (g) and m3 the crucible mass with the dry sample (g).

3.3.2 Protein content

The protein content was quantified in initial samples as described by the Dumas method (Saint-Denis and Goupy, 2004), using a conversion factor of nitrogen into protein of 5.0 in seaweed samples (Angell et al., 2016) and 6.25 in food products and microalgae. The analyser used was LECO FP-528, which performed the combustion of the samples of known mass in the presence of oxygen inside a high temperature chamber (900 ºC). To calibrate the standards, ethylenediamine tetraacetic acid (EDTA) was used. The nitrogen percentage of the samples was calculated based on thermal conductivity.

3.3.3 Total lipid content

The total lipid content was obtained using an adaption of the Folch Method (Folch et al., 1956), where 200 mg of each sample were mixed with 3 mL of a solution of chloroform and methanol (2:1, v/v). The samples were placed in a shaking water bath (25 ºC, 170 rpm) for 10 min and it was added 3 mL of hydrochloric acid (0.1 N) and 300 µL of magnesium chloride 0.5 % (p/v) Then, the samples were centrifuged (5 minutes, 4 ºC, 3000 × g), and the extraction of the organic phase was performed and filtered through a column filled with cotton and anhydrous sodium sulphate, to avoid the presence of residues and water, being this process repeated to extract the maximum amount of lipids. The process was repeated starting with the addition of the chloroform and methanol mixture with the only difference being the shaking time in the water bath (5 minutes). The second organic phase extracted was added to the corresponding phase previously collected. To evaporate the solvent, a nitrogen evaporator was used.

For the bioaccessible fraction, it was used a slightly modified Bligh and Dyer method (Bligh and Dyer, 1959). First, 4 mL chloroform and approximately 3 g of NaCl was added to the bioaccessible fraction followed by a homogenisation in a vortex and a centrifugation at 2000 × g for 10 minutes at 4 ºC. Then, the organic phase was collected and filtered through a column filled with cotton and anhydrous sodium sulphate. In the next step, 2 mL chloroform was added and the previous process repeated leading to a new organic phase extraction. The resulting phase was then evaporated in a rotary evaporator. The samples were weighed, solubilised in chloroform and stored at -20 ºC until further analysis. The total lipid content was calculated using the following equation:

$$\text{Total lipids } (\%) = \frac{\text{Mf} - \text{Mi}}{\text{S}} \times 100 \quad \textbf{(6)}$$

Where Mf corresponds to the final mass of the tube with lipids, Mi the initial tube mass and S the sample mass.

3.3.4 Carbohydrate content

The carbohydrate content was estimated by calculating the difference between 100 % and the sum of the moisture, ash, protein and total lipids contents.

3.4 Fatty acid profile by acid catalysis

The fatty acid profile was determined in initial and bioaccessible samples according to the methodology described by Bandarra et al. (1997), where fatty acid methyl esters (FAME's) were formed by acid-catalyzed transesterification. Briefly, 300 mg of freeze dried initial samples or 1.5 mL of bioaccessible lipids samples resuspended in chloroform, were mixed with 5 mL of an acetyl chloride:methanol (1:19) solution (prepared immediately before addition), vortexed and incubated in an 80 ºC water bath for 1 hour, according to the technique described by Lepage and Roy (1986), modified by Cohen et al. (1988). After cooling, it was added 1 mL of ultra-pure water and 2 mL of n-heptane to the samples, vortexed and centrifuged for 3 minutes at 4 ºC, 3000 × g. The organic phase was removed and filtered through a column filled with cotton and anhydrous sodium sulphate to a 2 mL vial.

Samples were applied to a DB-WAX (Agilent Technologies, Santa Clara, USA) capillary column (film thickness, 0.25 µm, 30 m × 0.25 mm i.d.), integrated in a Varian Star 3800 CP gas chromatograph (Walnut Creek, CA, USA), equipped with an auto sampler with a split injector (100:1) and a flame ionization detector, both at 250 ºC. The separation of the FAMEs was carried out with helium as the carrier gas and using a temperature program for the column starting at 180 ºC and increasing to 200 ºC at 4 ºC/minute, holding for 10 minutes at 200 ºC, heating to 210 ºC at the same rate, and holding at this temperature for 14.5 minutes. FAME's were identified by comparing their retention time with those of Sigma–Aldrich standards (PUFA-3, Menhaden oil, and PUFA-1, Marine source from Supelco Analytical).

Results were calculated in mg/g of edible part using a corrective factor based on thin-layer chromatography (TLC) lipid class analysis. Analyses were performed in triplicate.

3.5 Lipid class analysis (TLC)

The lipid classes were separated by analytical thin-layer chromatography (TLC), using an elution mixture of n-hexane, diethyl ether and formic acid (50:50:2 by volume), using a methodology described by Bandarra et al. (1997). Succinctly, the lipids extracted using the methods described in **3.3.3** were dissolved in chloroform with a final concentration of 10 mg/mL as well as the standards phospholipid (PL), monoacylglycerol (MAG), 1,3-diacylglycerol (DAG-1,3), 1,2-diacylglycerol (DAG-1,2), sterol (CH), free fatty acid (FFA) and triacylglycerol (TAG) (Sigma Chemical Co., St. Louis, Mo, USA). In order to start the analysis, plates coated with 0.25 mm silica gel G were placed in an oven for 1 hour at 110 ºC, removing water residues and the elution mixture was prepared, placed in a developing chamber and covered to begin the saturation phase. Then, 10 µL of the samples and standards were then applied in duplicate to the properly identified well in the plate and the plate was immersed in elution mixture inside a developing chamber. After the elution front reached the designed limit, plates were removed of the chamber, dried for 10 minutes, sprayed with 10 % phosphomolybdic acid in ethanol (w/v), and placed in an oven at 110 ºC for 10 minutes to visualize the lipids in the plate. The identification of lipid classes was performed by visual comparison with the standards and the quantification was calculated using a scanner and version 4.5.2 of Quantity One 1-D Analysis software from Bio-Rad (Hercules, CA, USA). This equipment allowed to calculate the relative percentages of the different identified lipid classes based on the area of the spots pondered by the optical density on the TLC plate.

3.6 Bioactive compounds

3.6.1 Preparation of Extracts

In order to determine the antioxidant activity and phenol content of the initial algae and food products, aqueous extracts of the samples were prepared using a 5 % (m/v) concentration. It was weighted 1.25 g of freeze dried sample, followed by addition of 25 mL of ultra-pure water and then homogenization with a Polytron PT 6100 (Kinematica, Luzern, Switzerland) during 1 minute at 30000 rpm. After that, the samples were placed in an orbital shaker (400 rpm) for 18 hours, centrifuged (10 minutes, 4 ºC, 3000 × g) and the supernatant collected to a tube, where it was kept at 4 ºC until further analysis.

To analyse the bioactive compounds of the bioaccessible fraction, the digested samples were homogenized in a vortex and used directly instead of preparing an extract.

3.6.2 Total Phenolic Content

The total phenolic content of the initial and bioaccessible samples was obtained through an adapted Folin-Ciocalteu method (Siriwoharn et al., 2004) using gallic acid as standard, as described in the work of Singleton and Rossi (1965). Accordingly, 100 µL of each extract or bioaccessible sample were added to a previously identified tube and mixed with 600 µL of ultra-pure water and 150 µL of a solution of water and Folin-Ciocalteu reagent (1:1). The samples were, then, allowed to stand for 5 min at room temperature and added 750 µL of a 2 % w/v sodium carbonate solution. After 1 hour and 30 minutes in the dark at room temperature, absorbance at 750 nm was measured in a Helios Alpha model (Unicam, Leeds, UK) UV-Vis spectrophotometer. The phenolic content was expressed as gallic acid equivalents (mg GAE/g) through the calibration curve of gallic acid (Sigma, Steinheim, Germany). For the bioaccessible samples, some interference by solutions and digestive enzymes may be relevant so, besides the method's blank, a bioaccessibility blank was measured and subtracted from the absorbance measured with the bioaccessible fraction samples.

3.6.3 Antioxidant Activity

The antioxidant activity was measured by using three distinctive methods, the determination of the radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Miliauskas et al., 2004), the determination of the radical scavenging activity of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) (Re et al., 1999) and the Ferric Ion Reducing Antioxidant Power (FRAP) technique where the antioxidant ability to reduce ferric-tripyridyl-triazine (Fe^{3+}-TPTZ) complex to the blue coloured ferrous form (Fe^{2+}) is assessed (Benzie and Strain, 1996).

3.6.3.1 DPPH

The radical scavenging activity of DPPH was determined spectrophotometrically. DPPH is considered a stable free radical because the spare electron is delocalized. When DPPH• reacts with an antioxidant compound, which can donate hydrogen, it is reduced, producing a change in colour (from deep—violet to light—yellow) that is measured at 515 nm on a UV/visible light spectrophotometer.

DPPH method procedure was initiated with the addition of 1 ml of the extracts prepared in **3.6.1** in triplicate to a previously identified tube. Then, 2 mL of DPPH (Sigma, Steinheim, Germany) 0.15 mM methanolic solution was added and thoroughly mixed. The samples were then placed in the dark at room temperature for 30 minutes and absorbance was measured at 517 nm in a Helios Alpha model (Unicam, Leeds, UK) UV/visible light spectrophotometer.

Results were expressed in mg of ascorbic acid equivalents (AA Eq) per g of dry weight of the samples.

3.6.3.2 ABTS

ABTS, 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation decolourisation test is a spectrophotometric method widely used for the assessment of antioxidant activity of various substances (Miliauskas et al., 2004). In this technique ABTS+• was generated by oxidation of ABTS with potassium persulfate.

ABTS radical scavenging activity measurement required the preparation of the reagent (7 mM ABTS+• solution), where 10 mg of ABTS was dissolved in 2.6 mL of a 2.45 mM potassium persulfate solution. The resultant solution was incubated for 16 hours in the dark at room temperature. After that, the ABTS+• solution was diluted with 5 mM sodium phosphate buffer (pH 7.4) until an absorbance value of 0.70 ± 0.02 at 734 nm was reached. Then, 20 µl of sample solutions prepared in 3.6.1 were mixed with 2 ml of the diluted ABTS+• solution, homogenized and incubated in the dark at 30 ºC for 6 minutes. The absorbance of the samples was measured at 734 nm in a Helios Alpha model (Unicam, Leeds, UK) UV/visible light spectrophotometer. The ABTS radical scavenging activity of the samples was expressed as µmol of trolox equivalents (Trolox Eq) per g of dry weight of the samples.

3.6.3.3 FRAP

In this assay, the colourless oxidized Fe^{3+} form of iron is converted to a blue-colored Fe^{2+} tri-pyridyl triazine (TPTZ)-reduced form, which is due to the action of the electron donation from antioxidants as illustrated in **Figure 5**. The assay measures a change in the absorbance at a wavelength of 593 nm.

The FRAP assay required the preparation the ferric-TPTZ reagent that comprised three solutions, 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM $FeCl_3 \bullet 6H_2O$ solution in the proportion 10:1:1 (v/v/v), respectively. Afterwards, 100 µL of the extracts prepared in 3.6.1, in triplicate, were mixed with 3 mL of the ferric-TPTZ reagent and incubated 30 minutes at 37 ºC in the dark. The absorbance readings were taken at 595 nm in a Helios Alpha model (Unicam, Leeds, UK) UV/visible light spectrophotometer, and the results are expressed in mM Fe (II).

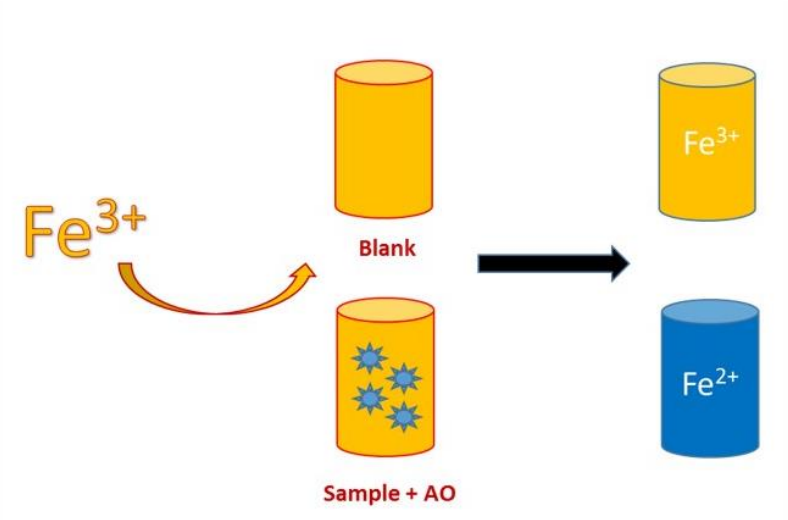

Figure 5 - Illustration of FRAP assay with the oxidation of Fe^{3+} form of iron to a blue coloured Fe^{2+}-TPTZ complex (GBiosciences, 2019).

3.7 Mineral Composition

The mineral composition of the initial and bioaccessible samples were determined using a methodology described by Moreira et al. (2015) with the purpose of evaluating the presence of chemical elements in samples. Before analysis, the samples had to undergo an acid digestion that required dried material, so the bioaccessible samples were placed in an oven for 12 hours and dried at 40 ºC. Between 0.3 and 0.5 g of freeze dried initial samples and dried bioaccessible samples were weighed in duplicate and 7.5 mL of nitric acid (HNO_3) at 65 % and 2.5 mL of hydrochloric acid (HCL) at 37 % was added. The samples were digested in a 115 minutes cycle (35 minutes to get to 45 ºC → 10 minutes at 45 ºC → 10 minutes to get to 90 ºC → 15 minutes at 90 ºC → 15 minutes to get to 105 ºC → 30 minutes at 105 ºC). After the digestion, the samples were cooled and diluted to 25 mL with ultra-pure water. The samples were then filtered and kept in labelled tubes.

The solutions were then analysed for lead (Pb), copper (Cu), iron (Fe), zinc (Zn), arsenic (As), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na) and phosphorus (P) by Inductively Coupled Plasma Atomic Emission Spectrometry using the equipment Thermo Scientific iCap 7000. This technique allows the quantification of various elements simultaneously through the use of high temperatures and resorting to calibration curves of each element.

3.8 Statistical Analysis

The statistical analysis of the data was performed using STATISTICA 10 (Stat-sof, Inc. USA, 2011), and the results were expressed as average ± standard deviation. To test the normality and the homogeneity of variance of data, the Kolmogorov-Smirnov's test and Cochran C-test, respectively, were used. Data which corroborated these assumptions were analysed by an one-way ANOVA distribution using the Tukey HSD to determine the difference in the constituents contents between the two algae species and between the food products or by a factorial ANOVA using the Tukey HSD to determine the difference the initial and bioaccessible samples. When normality and/or homogeneity of variance were not verified (Σ ω6 content in algae, 20:0, 22:0, 18 ω3 and Σ ω3 content in initial and bioaccessible samples of cookies and 18:0, 16:1 and ω3/ω6 ratio of initial an bioaccessible samples of sauces), data was tested non-parametrically with Kruskal–Wallis test (analysis of variance) followed by non-parametric multiple comparisons test. For all statistical tests the significance level (α) was 0.05.

4. Results and Discussion

4.1 Proximate Composition

4.1.1 Algae

The proximate composition of the studied marine organisms is displayed in **Table 2**, where the information regarding the seaweed *Cystoseira abies-marina* was provided by IPMA. The largest differences between the two species were observed in the protein and total lipids contents, where *Skeletonema sp.* presented a much higher value in both, reaching a content of 34.16 ± 0.01 g/100 g dw of protein and 10.21 ± 2.83 g/100 g dw of total lipids. The lipid content of species from the genus *Skeletonema* reported in most studies range from 13 to 30 % (Gao et al., 2019; Renaud et al., 1999; Rodolfi et al., 2009), however, the species *Skeletonema marinoi* is reported to have a lipid content of 9 % (D'Ippolito et al., 2015) which is similar to the results obtained in this species. The microalgae presented statistically higher values for almost all constituents except carbohydrates, with *Cystoseira* having 61.08 g per 100 g of dry weight. This macroalgae had very low lipid content (0.79 ± 0.13), hence further analysis regarding food products with *Cystoseira abies-marina* and fat content were not pursued. Other species belonging to the genus *Cystoseira* reported a lipid content that ranged from 4.2 to 8.6 g/100 g dw, (Fariman et al., 2016; Terasaki et al., 2009), which was not obtained in this species.

Table 2 - Proximate composition (moisture, ash, protein, total fat and carbohydrates) measured in the species used in the food products. Values are presented as average ± standard deviation. Different lowercase letters within a column correspond to statistical differences ($p<0.05$) between the species.

	Moisture (%)	Ash (g/ 100 g dw)	Protein (g/ 100 g dw)	Total Lipids (g/ 100 g dw)	Carbohydrates (g/ 100 g dw)
Skeletonema sp.	8.58 ± 0.05^{b}	32.16 ± 0.04^{b}	34.16 ± 0.01^{b}	10.21 ± 2.83^{b}	14.89
***C. abies-marina*[1]**	6.22^{a}	25.20 ± 0.05^{a}	6.71 ± 0.16^{a}	0.79 ± 0.13^{a}	61.08

[1] Values provided by IPMA

4.1.2 Bioaccessible lipid fraction of *Skeletonema* sp.

The bioaccessible lipid assay was performed on the freeze dried microalgae *Skeletonema sp.* only, using an *in vitro* digestion model. Although this species had roughly 10 % of lipids, the results showed that no fat was bioaccessible, thus no further results regarding this lipid fraction were considered. An explanation for this could be that the used *in vitro* method was not appropriate to the algae matrix and couldn't extract the lipids properly. Alternatively, it can be assumed that the cell wall and other membranous structures in *Skeletonema* sp. retained

the lipid fraction and rendered it inaccessible for intestinal absorption. Furthermore, a study Bonfanti et al. (2018) about bioaccessibility of lipids in the microalgae *Isochrysis galbana*, reported a low lipid bioaccessibility between 7-15 %, which could mean that the lipid bioaccessibility in microalgae is limited.

4.1.3 Food products

The proximate composition of the food products is displayed in **Table 3** for the cookies and **Table 4** for the sauces. Observing the results only moisture and ash showed significant differences between the three cookies. Regarding taste, it is worth noting that while cookies enriched in *Skeletonema* sp. were considered unpleasant, cookies with incorporated *C. abies-marina* were deemed pleasant and similar to the control cookies. The protein content was shown not to have significant differences between cookies which is unexpected considering the high amount of protein of *Skeletonema* sp*.* (34.16 ± 0.01 g/100 g dw)*.* This could mean that a higher amount of algal biomass is needed in the cookies to make a noticeable difference, however the sensory aspects of the cookie would be greatly affected. Concerning the sauces, it was not observed any differences between the three sauces in any case, proving that it is necessary to use a higher concentration of algal biomass to notice any variation. Comparing cookies *vs* sauces, besides the obvious difference in moisture content, it should be noted that the sauces possess the double protein content of the cookies.

It is relevant to compare the nutritional value calculated in **3.1.3** to the obtained results since there is some similarities. The lipid content obtained per fresh weight of control cookie is 26.6 g/100 g, the protein content is 9.7 g/100 g and the carbohydrate content is 60.4 g/100 g. Per fresh weight the lipid content of control sauce is 4.0 g/100 g, the protein content is 3.4 g/100 g and the carbohydrate content is 6.7 g/100 g. The obtained amount of lipids, protein and carbohydrates of the cookies was a bit higher than the calculated however the nutritional value of the sauces exhibit similarities to the calculated in the nutrition label.

Table 3 - Proximate composition (moisture, ash, protein, total lipids and carbohydrates) measured in the cookies prepared. Values are presented as average ± standard deviation. Different lowercase letters within a column correspond to statistical differences ($p<0.05$) between the cookies.

	Moisture (%)	**Ash (g/ 100 g dw)**	**Protein (g/ 100 g dw)**	**Total Lipids (g/ 100 g dw)**	**Carbohydrates (g/ 100 g dw)**
Control Cookie	1.59 ± 0.15^{a}	1.80 ± 0.05^{a}	9.85 ± 0.50^{a}	27.00 ± 1.16^{a}	61.35
***C. abies-marina* 3% Cookie**	3.81 ± 0.87^{b}	2.55 ± 0.02^{b}	9.96 ± 1.20^{a}	-	-
***Skeletonema sp.* 3% Cookie**	1.65 ± 0.13^{a}	2.72 ± 0.03^{c}	10.83 ± 0.34^{a}	30.47 ± 2.08^{a}	55.98

Table 4 - Proximate composition (moisture, ash, protein, total lipids and carbohydrates) measured in the sauces prepared. Values are presented as average ± standard deviation. Different lowercase letters within a column correspond to statistical differences ($p<0.05$) between the sauces.

	Moisture (%)	Ash (g/ 100 g dw)	Protein (g/ 100 g dw)	Total Lipids (g/ 100 g dw)	Carbohydrates (g/ 100 g dw)
Control Sauce	85.68 ± 0.04[a]	9.56 ± 0.19[a]	23.52 ± 1.71[a]	28.08 ± 1.35[a]	38.84
***C. abies-marina* 2% Sauce**	85.25 ± 0.12[a]	12.29 ± 0.86[a]	21.03 ± 1.37[a]	-	-
***Skeletonema sp.* 2% Sauce**	84.78 ± 0.39[a]	11.06 ± 1.42[a]	24.09 ± 0.13[a]	26.93 ± 1.34[a]	37.92

4.1.4 Bioaccessible lipid fraction of food products

The *in vitro* digestion model was performed for the control products and the products containing *Skeletonema* sp., in order to assess the bioaccessible lipid fraction and are displayed in **Table 5**. For this assay, it was used fresh (not freeze dried) material and, therefore, the before and after *in vitro* digestion results are presented in g per 100 g of edible part. The total lipid content decreased in the bioaccessible fraction in every product except *Skeletonema sp.* sauce, which exhibit a similar content to the initial sample. Observing the bioaccessibility percentage in **Figure 6**, over 60% of the lipid fraction in the control cookie were bioaccessible, with the two cookies having no differences regarding bioaccessible lipids. Regarding the sauces, the results show that over 80% of the lipid content is bioaccessible in both sauces, with *Skeletonema* sp. sauce having a higher bioaccessible lipid fraction than both cookies. These percentages are in accordance with other studies reporting a good digestion of the lipid fraction, with 53-80 % of bioaccessible lipids (Afonso et al., 2017; Costa et al., 2016, 2015).

Table 5 - Total lipids obtained before (Initial) and after *in vitro* digestion (Bio) of control and *Skeletonema* sp. cookie and sauce calculated in g of 100 g of food. Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences (p<0.05) between the initial and bioaccessible lipids of the cookies. Different uppercase letters within a row correspond to statistical differences (p<0.05) between initial and bioaccessible lipids of the sauces.

		Total lipids (g/100g)
Control Cookie	**Initial**	26.57 ± 1.14^{b}
	Bio	18.06 ± 2.84^{a}
***Skeletonema sp.* 3 % Cookie**	**Initial**	29.97 ± 2.05^{b}
	Bio	15.98 ± 2.97^{a}
Control Sauce	**Initial**	4.02 ± 0.19^{B}
	Bio	3.21 ± 0.36^{A}
***Skeletonema sp.* 2 % Sauce**	**Initial**	4.10 ± 0.20^{B}
	Bio	3.53 ± 0.03^{AB}

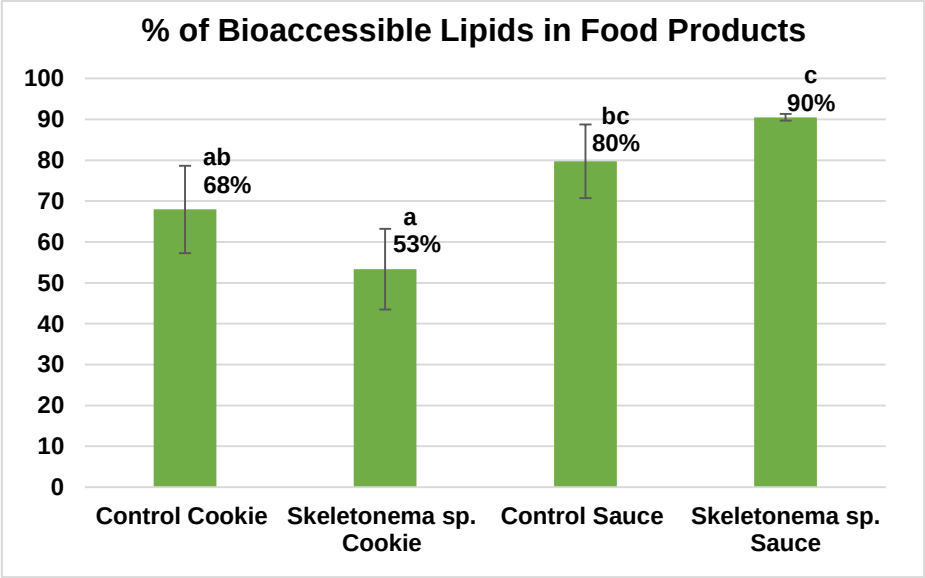

Figure 6 - Bioaccessibility (%) of total lipids obtained in control and *Skeletonema* sp. food products Values are presented as average ± standard deviation. Different lowercase letters represent values statistically different between bioaccessibility percentages ($p < 0.05$).

4.2 Fatty Acid Profile

4.2.1 Algae

The fatty acid profile obtained by acid catalysis in microalgae *Skeletonema* sp. and macroalgae *Cystoseira abies-marina* (in percentage of total fatty acids and in mg/g dry weight)

is shown in **Table 6**. Observing the results, there are important differences and similarities between the two algae species. Regarding the percentage of total fatty acids, both species had similar SFA content, corresponding to nearly half of the lipid content. The most abundant saturated fatty acid, and overall fatty acid, was myristic acid (14:0) in *Skeletonema* sp. case and palmitic acid (16:0) in *C. abies-marina*. The microalgae presented a higher percentage of Monounsaturated FA's (MUFA) than the seaweed, but again there were different major MUFA's in both species, with *Skeletonema sp.* having 27.85 ± 0.33 % of palmitoleic acid (16:1) and *C. abies-marina* possessing 18.39 ± 0.25 % of oleic acid (18:1).

Concerning PUFA, the brown seaweed showed a much higher percentage of these FAs, mainly ω6 PUFA, with the ω3/ω6 ratio showing significant differences between the species. The high content of PUFA's on the genus *Cystoseira* has been reported in several studies (Airanthi et al., 2011; Terasaki et al., 2009). Although present in a lower total percentage, the microalgae showed an interesting content of ω3 PUFA, with important FA such as eicosapentaenoic acid (EPA, 22:6 ω3) and docosahexaenoic acid (DHA, 20:5 ω3) present in a higher percentage than in *C. abies-marina*. Arachidonic acid (AA, 20:4 ω6) was the major PUFA present in the seaweed reaching 15.23 ± 0.50 % of the total fatty acids. This percentage of AA was also reported in a study with *Cystoseira hakodatensis* by Airanthi et al. (2011). Alfa-linolenic acid (ALA, 18:3 ω3) and linoleic acid (18:2 ω6) also had high percentages in this species. Coincidently, the first two mentioned PUFA were not detected in the microalgae and linoleic acid was present in a very low percentage (0.59 ± 0.00 %). The most abundant PUFA in *Skeletonema* sp. was 16:3 ω4 with a percentage of 7.21 ± 0.19 %. Studies with a species from the genus *Skeletonema* (Berge et al., 1995; Cardoso et al., unpublished.; Zhukova and Aizdaicher, 1995) shows that the main PUFAs found were 16:3 ω4 and DHA (20:5 ω3), the main MUFA was 16:1 and the predominant SFA's were 14:0 and 16:0 which is in agreement with the obtained results. However, it is reported that the dominant fatty acids in species from the genus Skeletonema are PUFA's, something that was not achieved in this study. Fariman et al. (2016) studied the fatty acid profile of *Cystoseira indica* reaching similar results regarding the major fatty acids present in *C. abies-marina* with differences ω3 fatty acid content which was lower in this study.

As stated before, *Skeletonema* sp. has a much higher percentage of lipids that *C. abies-marina* with this being accentuated by comparing the mg of FAME present in a gram of each species. Consequently, *Skeletonema sp.* displayed higher FA absolute content for the most part of the fatty acids. In *C. abies-marina,* oleic acid (18:1) was the only FA that exhibit approximately the same absolute content in both species and concerning the sum of ω6 PUFA, *Cystoseira* displayed a higher absolute content than the microalgae.

The bioaccessible fraction of FAs of *Skeletonema* sp. was calculated and was determined to be 0 %, therefore the results are not displayed in the table.

Table 6 - Fatty acid profile of the studied species *Skeletonema* sp. and *Cystoseira abies-marina* (in % of total FA and mg/g of dry weight). Values are presented as average ± standard deviation, where N. d. – not detected. Different lowercase letters within a row correspond to statistical differences (p<0.05) between the relative (%) of total FA between algae. Different uppercase letters within a row correspond to statistical differences (p<0.05) between the absolute (mg/ g dw) FA of algae.

FATTY ACID	*Skeletonema sp.*		*C. abies-marina*[1]	
	% of Total FA	mg/g dw	% of Total FA	mg/g dw
14:0	31.47 ± 1.54^{b}	26.67 ± 1.30^{B}	6.48 ± 0.24^{a}	0.41 ± 0.01^{A}
15:0 isobr	1.02 ± 0.06^{b}	0.87 ± 0.05^{B}	N. d.^{a}	N. d.^{A}
15:0	0.92 ± 0.02^{b}	0.78 ± 0.02^{B}	N. d.^{a}	N. d.^{A}
16:0	10.51 ± 0.18^{a}	8.91 ± 0.16^{B}	34.27 ± 1.32^{b}	2.04 ± 0.08^{A}
17:0 isobr	0.93 ± 0.95^{b}	0.79 ± 0.04^{B}	N. d.^{a}	N. d.^{A}
18:0	N. d.^{a}	N. d.^{A}	0.86 ± 0.03^{b}	0.05 ± 0.00^{B}
21:0	N. d.^{a}	N. d.^{A}	1.11 ± 0.03^{b}	0.07 ± 0.00^{B}
Σ SFA	46.27 ± 1.29^{a}	39.21 ± 1.09^{B}	46.88 ± 0.22^{a}	2.78 ± 0.01^{A}
16:1 (16:1ω9 + 16:1ω7)	27.85 ± 0.33^{b}	23.60 ± 0.28^{B}	2.30 ± 0.03^{a}	0.14 ± 0.00^{A}
18:1 (18:1ω9 + 18:1ω7)	1.46 ± 0.11^{a}	1.24 ± 0.10^{A}	18.39 ± 0.25^{b}	1.09 ± 0.02^{A}
20:1 (20:1 ω11 + 20:1ω9 + 20:1ω7)	N. d.^{a}	N. d.^{A}	0.25 ± 0.11^{b}	0.01 ± 0.01^{B}
Σ MUFA	29.51 ± 0.47^{b}	25.01 ± 0.40^{B}	21.03 ± 0.14^{a}	1.25 ± 0.01^{A}
16:2ω4	4.25 ± 0.16^{b}	3.60 ± 0.14^{B}	N. d.^{a}	N. d.^{A}
16:3ω4	7.21 ± 0.19^{b}	6.11 ± 0.16^{B}	0.14 ± 0.02^{a}	0.01 ± 0.00^{A}
16:4ω3	1.49 ± 0.04^{b}	1.26 ± 0.03^{B}	N. d.^{a}	N. d.^{A}
18:2ω6	0.59 ± 0.00^{a}	0.50 ± 0.00^{B}	5.70 ± 0.05^{b}	0.01 ± 0.00^{A}
18:3ω3	N. d.^{a}	N. d.^{A}	4.49 ± 0.03^{b}	0.27 ± 0.00^{B}
18:4ω3	1.45 ± 0.06^{b}	1.23 ± 0.05^{B}	1.19 ± 0.02^{a}	0.07 ± 0.00^{A}
20:4ω6	N. d.^{a}	N. d.^{A}	15.23 ± 0.50^{b}	0.90 ± 0.03^{B}
20:4 ω3	N. d.^{a}	N. d.^{A}	0.62 ± 0.05^{b}	0.04 ± 0.00^{B}
20:5ω3	4.20 ± 0.29^{b}	3.56 ± 0.25^{B}	1.87 ± 0.10^{a}	0.11 ± 0.01^{A}
22:6ω3	0.58 ± 0.11^{b}	0.49 ± 0.10^{B}	N. d.^{a}	N. d.^{A}
Σ PUFA	19.58 ± 0.97^{a}	16.59 ± 0.82^{B}	30.22 ± 0.59^{b}	1.80 ± 0.04^{A}
Σ ω3	7.53 ± 0.64^{a}	6.38 ± 0.54^{B}	8.49 ± 0.16^{a}	0.50 ± 0.01^{A}
Σ ω6	0.59 ± 0.00^{a}	0.50 ± 0.00^{A}	21.42 ± 0.49^{b}	1.27 ± 0.03^{B}
ω3/ ω6	12.68 ± 1.10^{b}	12.68 ± 1.10^{B}	0.40 ± 0.00^{a}	0.40 ± 0.00^{A}

[1] Values provided by IPMA

4.2.2 Food products

The FA profile obtained by acid catalysis in the control cookie and the cookie with 3 % of *Skeletonema sp.* (in % of total fatty acids and in mg/g dry weight) is shown in **Table 7**. In terms of percentage there were no significant differences between the cookies except for palmitoleic acid (16:1) that is present in a higher percentage in *Skeletonema* sp. cookie, which falls in line with the high 16:1 percentage found in this microalgae (**Table 6**). This means that, to observe larger significant changes, it is required a higher percentage of microalgae added to the cookie. Nevertheless, both cookies exhibit high percentages of PUFAs, 44.49 ± 0.24 % in control cookie and 43.91 ± 1.23 % in microalgae cookie, being the most abundant class of FAs. Linoleic acid (18:2 ω6) corresponds to the largest share of the percentage of PUFA and overall percentage of FAs. ALA (18:3 ω3), an essential FA, is present at a relevant percentage making about 5% of the total FAs in both cookies. Concerning SFA, the most abundant in the cookies is 16:0 palmitic acid, comprising 17% of the total FAs. The second most abundant FA present in this food is 18:1, oleic acid, reaching values of 29.01 ± 1.21 and 28.42 ± 0.87 % in control and *Skeletonema sp.* cookies, respectively, which is not unexpected, considering that it's a very common, present in most cookies ingredients. Margarine was the ingredient used in the highest amount in the cookies. Nadeem *et al*., (2017) studied the fatty acid profile of margarines, where 16:0, 18:0, 18:1 and 18:2 were the most abundant, which is represented as well in the fatty acid profile of the cookies obtained.

Regarding the FA absolute contents (mg/g dw), there were significant differences between the two cookies. Starting by addressing the saturated FAs, the global mass of this class of FA was similar in both cookies, with differences in the amount of specific SFAs. The cookie with *Skeletonema* sp. displayed a higher absolute content of myristic acid (14:0) than the control cookie, which can be explained by the fact that it is the most abundant FA found in the microalgae (**Table 6**). Palmitic and stearic acids (16:0 and 18:0, respectively) are also presented at a higher content in *Skeletonema* sp. cookie. Analysing MUFAs the results were mostly similar between the two cookies, with only palmitoleic acid (16:1) being superior in *Skeletonema* sp. cookie, which is consistent with its high content in the microalgae. Concerning PUFA, whose major components are linoleic acid (18:2 ω6) and ALA (18:3 ω3), the results show that more than 100 mg are found in 1 gram of dry weight of both cookies which is a considerable amount. The ω3/ω6 ratio is low mainly because, the amount of linolenic acid is much higher than that of any of the ω3 components.

Table 7 - Fatty acid profile of the cookies prepared (Control and with 3 % of *Skeletonema* sp.) presented in % of total FA and mg/g of dry weight. Values are presented as average ± standard deviation, where N. d. – not detected. Different lowercase letters within a row correspond to statistical differences (p<0.05) between the % of total FA between the cookies. Different uppercase letters within a row correspond to statistical differences (p<0.05) between the FA mass (mg/ g dw) of the cookies.

FATTY ACID	Control Cookie		3 % SKT Cookie	
	% of Total FA	mg/g dw	% of Total FA	mg/g dw
12:0	2.74 ± 0.81^{a}	6.56 ± 1.95^{A}	3.17 ± 1.04^{a}	8.17 ± 2.69^{A}
14:0	1.10 ± 0.11^{a}	2.63 ± 0.25^{A}	1.58 ± 0.21^{b}	4.08 ± 0.54^{B}
16:0	17.06 ± 0.17^{a}	40.90 ± 0.40^{A}	17.13 ± 0.12^{a}	44.12 ± 0.31^{B}
18:0	3.60 ± 0.10^{a}	8.64 ± 0.23^{A}	3.63 ± 0.07^{a}	9.34 ± 0.19^{B}
Σ SFA	25.23 ± 0.95^{a}	60.47 ± 2.28^{A}	26.07 ± 1.79^{a}	67.18 ± 4.62^{A}
16:1 (16:1ω9 + 16:1ω7)	0.15 ± 0.01^{a}	0.35 ± 0.02^{A}	0.58 ± 0.04^{b}	1.48 ± 0.11^{B}
18:1 (18:1ω9 + 18:1ω7)	29.01 ± 1.21^{a}	69.55 ± 2.91^{A}	28.42 ± 0.87^{a}	73.21 ± 2.24^{A}
20:1 (20:1 ω11 + 20:1ω9 + 20:1ω7)	0.12 ± 0.00^{b}	0.28 ± 0.01^{B}	N. d.^{a}	N. d.^{A}
Σ MUFA	29.19 ± 1.33^{a}	69.98 ± 3.19^{A}	28.80 ± 1.17^{a}	74.20 ± 3.00^{A}
18:2ω6	38.88 ± 0.14^{a}	93.21 ± 0.34^{A}	38.37 ± 1.03^{a}	98.87 ± 2.66^{B}
18:3ω3	5.52 ± 0.36^{a}	13.23 ± 0.86^{A}	5.54 ± 0.23^{a}	14.28 ± 0.60^{A}
Σ PUFA	44.49 ± 0.24^{a}	106.64 ± 0.58^{A}	43.91 ± 1.23^{a}	113.14 ± 3.17^{B}
Σ ω3	5.52 ± 0.36 ^{a}	13.23 ± 0.86^{A}	5.54 ± 0.23^{a}	14.28 ± 0.60^{A}
Σ ω6	38.97 ± 0.21^{a}	93.41 ± 0.51^{A}	38.37 ± 1.03^{a}	98.87 ± 2.66^{B}
ω3/ω6	0.14 ± 0.01^{a}	0.14 ± 0.01^{A}	0.14 ± 0.00^{a}	0.14 ± 0.00^{A}

The FA profile determined by acid catalysis in control and with 2 % of *Skeletonema* sp. sauce (in % of total FAs and in mg/ g dry weight) is displayed in **Table 8.** Similar to the FA profile in the cookies, there were not many significant differences between the control and *Skeletonema* sp.-containing foods regarding the % of total FAs. The most notorious differences were related to the content of myristic acid (14:0) and palmitoleic acid (16:1), which were higher in *Skeletonema* sp. sauce due to its enrichment in this microalgae. Observing the results, the overwhelming amount of MUFAs present in both sauces is the most outstanding feature, making up 70.17 ± 0.85 % on the control sauce and 69.83 ± 0.06 % of the total FAs of microalgae sauce. Of this percentage, almost all is oleic acid (18:1), an expected result, since one of the main ingredients used in the preparation of the sauce was olive oil, known for having

a big percentage of oleic and linoleic acid (Negro et al., 2019). Onion is a major ingredient used in the sauces and its main fatty acids are oleic (18:1), palmitic (16:0) and linolenic acid (18:3 ω3), which are also present in high quantities in the sauce (Bello et al., 2013). Saturated FAs make up 18.29 ± 0.58 % and 18.51 ± 0.19 % on the control and *Skeletonema sp.* sauce, with the most abundant being 16:0, palmitic acid. PUFAs had a low representation with linoleic acid (18:2 ω6) being the most important of this class of FAs with a percentage of 10.43 ± 0.36 and 10.28 ± 0.18 % of the total FA's of control and microalgae sauce, respectively, leading to a very low ω3/ω6 ratio.

Table 8 - Fatty acid profile of the sauces prepared (Control and with 2 % of *Skeletonema* sp.) presented in % of total FA and mg/g of dry weight. Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences (p<0.05) between the % of total FA. Different uppercase letters within a row correspond to statistical differences (p<0.05) between the FA mass (mg/ g dw) of the sauces.

FATTY ACID	Control Sauce		2% SKT Sauce	
	% of Total FA	mg/g dw	% of Total FA	mg/g dw
14:0	0.17 ± 0.04^{a}	0.43 ± 0.10^{A}	0.74 ± 0.03^{b}	1.75 ± 0.08^{B}
16:0	14.46 ± 0.83^{a}	36.68 ± 2.10^{A}	14.11 ± 0.33^{a}	33.21 ± 0.78^{A}
18:0	3.02 ± 0.06^{a}	7.66 ± 0.14^{B}	2.96 ± 0.04^{a}	6.96 ± 0.09^{A}
Σ SFA	18.29 ± 0.58^{a}	46.39 ± 1.46^{B}	18.51 ± 0.19^{a}	43.58 ± 0.44^{A}
16:1 (16:1ω9 + 16:1ω7)	1.27 ± 0.06^{a}	3.23 ± 0.15^{A}	1.87 ± 0.01^{b}	4.41 ± 0.03^{B}
18:1 (18:1ω9 + 18:1ω7)	68.68 ± 0.86^{a}	174.20 ± 2.10^{B}	67.74 ± 0.06^{a}	159.49 ± 0.15^{A}
20:1 (20:1 ω11 + 20:1ω9 + 20:1ω7)	0.22 ± 0.02^{a}	0.56 ± 0.05^{A}	0.21 ± 0.01^{a}	0.50 ± 0.02^{A}
Σ MUFA	70.17 ± 0.85^{a}	177.99 ± 2.15^{B}	69.83 ± 0.06^{a}	164.41 ± 0.14^{A}
18:2ω6	10.43 ± 0.36^{a}	26.46 ± 0.90^{B}	10.28 ± 0.18^{a}	24.20 ± 0.42^{A}
18:3ω3	0.76 ± 0.05^{a}	1.94 ± 0.13^{A}	0.77 ± 0.02^{a}	1.81 ± 0.04^{A}
Σ PUFA	11.43 ± 0.42^{a}	28.99 ± 1.06^{B}	11.32 ± 0.20^{a}	26.66 ± 0.47^{A}
Σ ω3	0.76 ± 0.05^{a}	1.94 ± 0.13^{A}	0.83 ± 0.06^{a}	1.96 ± 0.14^{A}
Σ ω6	10.43 ± 0.36^{a}	26.46 ± 0.90^{B}	10.28 ± 0.18^{a}	24.20 ± 0.42^{A}
ω3/ω6	0.07 ± 0.00^{a}	0.07 ± 0.00^{A}	0.08 ± 0.01^{a}	0.08 ± 0.01^{A}

Analysing the FA absolute content (mg/g dw) present in both foods, there were some significant differences worth mentioning. As noted in the % of total FAs, the microalgae sauce also showed a higher content of myristic acid (14:0) and palmitoleic acid (16:1). The control

sauce displayed a higher content of the SFA stearic acid (18:0), the MUFA oleic acid (18:1), and the PUFA linoleic acid (18:2 ω6), all of which were not detected in the microalgae or were present at very low levels. These differences were large enough to produce significant differences in the total absolute content of the 3 classes of FAs, SFAs, MUFAs, and PUFAs, between the control sauce and the *Skeletonema* sp. sauce.

4.2.3 Bioaccessible FAME fraction of food products

The *in vitro* digestion model performed on the control and *Skeletonema* sp.-containing food products allowed to assess the bioaccessible FAME fraction. As stated before, for this assay it was used fresh (non-freeze dried) material and, therefore, the before and after *in vitro* digestion results are presented in g per 100 g edible part.

For the bioaccessible FAME of the cookies, the results are displayed in **Table 9** and the percentage of bioaccessibility of major FAME's is presented in **Figure 7**.Observing the results regarding the concentration of FAME in initial and bioaccessible samples, it can be concluded that, for the most part, the FAME of the control cookie were more bioaccessible than the FAME of the cookie with the microalgae, which is compatible with the fact that *Skeletonema* sp. fat was not bioaccessible. In some cases, SFAs, such as tridecylic acid (13:0) and behenic acid (22:0) were detected in higher amounts in the bioaccessible samples, although in very low quantities still. This could mean that the *in vitro* digestion model managed to make these FAs more available to be detected. The two major FAs present in the cookies, the MUFA oleic acid (18:1) and PUFA linoleic acid (18:2 ω6) presented the highest bioaccessible fraction of all the FAs in both cookies. Overall the MUFAs were the fatty acids that showed the most positive results regarding bioaccessibility, which was observed in other studies that reported a high quantities of total MUFA and oleic acid in the bioaccessible fraction (Gomes et al., 2019).

The percentage of bioaccessibility showed more clearly that FAMEs in the control cookie were more bioaccessible than in the *Skeletonema*-enriched cookie. MUFA's oleic acid and palmitoleic acid showed the highest bioaccessibility percentages with 81 % and 90 %, respectively in the control cookie. In the *Skeletonema* cookie the highest bioaccessibility was achieved also in oleic acid with 71 % and linoleic acid (18:2 ω6) with 65 %. From a nutritional point of view, it is relevant to point out that while MUFA and PUFA achieved high bioaccessibility percentages, saturated fatty acids, which are associated with a higher risk of cardiovascular diseases, showed low bioaccessibility. Literature reports a much higher bioaccessibility percentage of SFA (60-88 %) (Costa et al., 2015; Gomes et al., 2019) than the obtained in the cookies (30-39 %).

Table 9 - Fatty acid profile before (Initial) and after *in vitro* digestion (Bio) of Control Cookie and 3 % *Skeletonema* sp. cookie, presented in mg of fatty acid per g of cookie. Values are presented as average ± standard deviation, where N. d. – not detected. Different lowercase letters within a row correspond to statistical differences (p<0.05) between the initial and bioaccessible FA mass (mg/ g) of the cookies.

FATTY ACID	Control Cookie		3 % *Skeletonema sp.* Cookie	
	Initial (mg/g)	Bio (mg/g)	Initial (mg/g)	Bio (mg/g)
12:0	6.45 ± 0.00	N. d.	8.03 ± 0.00	N. d.
13:0	0.92 ± 0.00^{a}	3.17 ± 0.79^{c}	1.75 ± 0.00^{ab}	2.42 ± 0.08^{bc}
14:0	2.58 ± 0.00^{b}	1.22 ± 0.14^{a}	4.01 ± 0.00^{c}	1.11 ± 0.02^{a}
16:0	40.25 ± 0.00^{c}	15.90 ± 0.55^{b}	43.40 ± 0.00^{d}	13.50 ± 0.00^{a}
18:0	8.50 ± 0.00^{c}	2.45 ± 0.07^{b}	9.19 ± 0.00^{d}	1.92 ± 0.11^{a}
20:0	0.32 ± 0.00^{b}	0.25 ± 0.00^{ab}	N. d.^{a}	0.20 ± 0.00^{ab}
22:0	0.38 ± 0.00^{a}	0.50 ± 0.02^{ab}	0.82 ± 0.00^{b}	0.45 ± 0.01^{ab}
Σ SFA	59.51 ± 0.00^{c}	27.61 ± 1.41^{b}	66.07 ± 0.00^{d}	19.66 ± 0.54^{a}
16:1 (16:1ω9 + 16:1ω7)	0.23 ± 0.00^{a}	0.21 ± 0.03^{a}	1.46 ± 0.00^{c}	0.42 ± 0.02^{b}
18:1 (18:1ω9 + 18:1ω7)	68.44 ± 0.00^{c}	55.58 ± 0.89^{b}	72.01 ± 0.00^{d}	51.10 ± 0.10^{a}
20:1 (20:1 ω11 + 20:1ω9 + 20:1ω7)	0.28 ± 0.00	0.09 ± 0.00	N. d.	0.09 ± 0.00
Σ MUFA	68.86 ± 0.00^{c}	58.65 ± 0.93^{b}	72.98 ± 0.00^{d}	51.71 ± 0.16^{a}
16:3ω4	N.d.^{a}	0.04 ± 0.01^{b}	N.d.^{a}	0.03 ± 0.01^{b}
18:2ω6	91.73 ± 0.00^{c}	71.64 ± 0.73^{b}	97.23 ± 0.00^{d}	62.97 ± 0.42^{a}
18:3ω6	0.20 ± 0.00^{b}	0.26 ± 0.05^{b}	N.d.^{a}	0.20 ± 0.00^{b}
18:3ω3	13.02 ± 0.00^{ab}	9.56 ± 0.26^{ab}	14.04 ± 0.00^{b}	8.32 ± 0.14^{a}
Σ PUFA	104.95 ± 0.00^{c}	85.03 ± 0.73^{b}	111.28 ± 0.00^{d}	71.24 ± 0.36^{a}
Σ ω3	13.02 ± 0.00^{ab}	9.63 ± 0.27^{ab}	14.04 ± 0.00^{b}	8.30 ± 0.16^{a}
Σ ω6	91.93 ± 0.00^{c}	75.35 ± 0.53^{b}	97.23 ± 0.00^{d}	62.84 ± 0.37^{a}
ω3/ω6	0.14 ± 0.00^{b}	0.13 ± 0.00^{a}	0.14 ± 0.00^{b}	0.13 ± 0.00^{a}

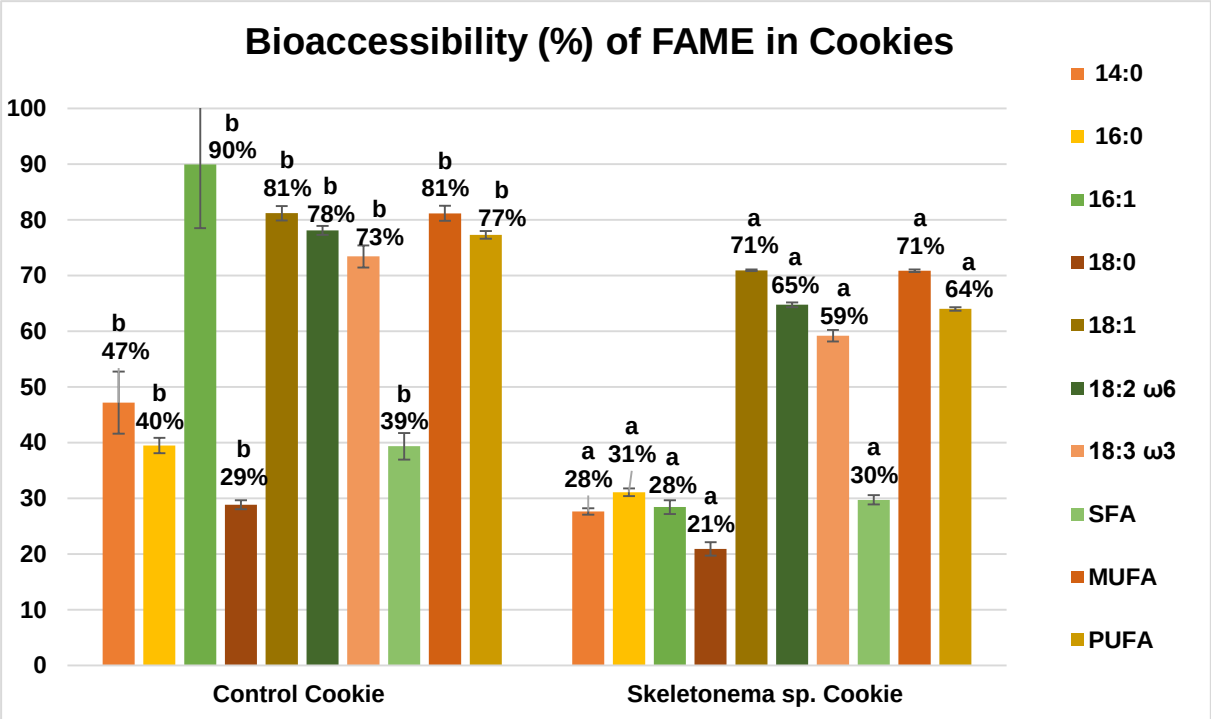

Figure 7 - Bioaccessibility (%) of major FAME's obtained in control and *Skeletonema* sp. food products Values are presented as average ± standard deviation. Different lowercase letters represent values statistically different between bioaccessibility percentages ($p < 0.05$) of control and *Skeletonema* sp. cookie.

Concerning the bioaccessibility of FAMEs in the sauces, for a comparison between initial and bioaccessible fraction results are shown in **Table 10** and the bioaccessibility percentages are shown in **Figure 8**. Analysing initial a bioaccessible fraction, there was a reduction in the bioaccessible fraction for almost all FAME's. SFAs showed the biggest decrease in the bioaccessible fraction. Comparing the two bioaccessible fractions of the sauces only 14:0, 16:3 ω4 and total MUFA's showed differences between sauces with diatom enriched sauce presenting higher values in the digested fraction. Once again, the monounsaturated FAs presented a higher value in bioaccessible fraction, with oleic acid (18:1) corresponding to almost the entire MUFA bioaccessible fraction in both food products. The PUFA detected in highest amount in bioaccessible fraction was linoleic acid (18:2 ω6).

The bioaccessibility percentages of control and *Skeletonema* sp. sauce showed no differences with the exception of MUFA that had a higher bioaccessibility in the microalgae sauce. Considering that the lipids of *Skeletonema* sp. were not bioaccessible, the mixture between the ingredients of the sauce and the microalgae proved to be beneficial in MUFA bioaccessibility. The SFAs presented the lowest bioaccessibility percentages (38-53 %) and MUFA's the highest (86-95 %) similar to what was observed in the cookies. 18:0 showed particularly low percentages which is not in agreement with other studies (Costa et al., 2015).

Table 10 - Fatty acid profile before (Initial) and after *in vitro* digestion (Bio) of Control Sauce and 2 % *Skeletonema* sp. Sauce, presented in mg of fatty acid per g of sauce. Values are presented as average ± standard deviation, where N. d. – not detected. Different lowercase letters within a row correspond to statistical differences (p<0.05) between the initial and bioaccessible FA mass (mg/ g) of the sauces.

FATTY ACID	Control Sauce		2 % *Skeletonema sp.* Sauce	
	Initial (mg/g)	Bio (mg/g)	Initial (mg/g)	Bio (mg/g)
14:0	0.06 ± 0.00^{a}	0.05 ± 0.00^{a}	0.27 ± 0.00^{b}	0.22 ± 0.05^{b}
16:0	5.25 ± 0.00^{c}	2.53 ± 0.65^{a}	5.06 ± 0.00^{bc}	3.35 ± 0.57^{ab}
18:0	1.10 ± 0.00^{b}	0.11 ± 0.06^{a}	1.06 ± 0.00^{b}	0.13 ± 0.00^{a}
20:0	0.14 ± 0.00^{b}	0.13 ± 0.02^{ab}	0.14 ± 0.00^{b}	0.12 ± 0.00^{a}
Σ SFA	6.64 ± 0.00^{b}	2.50 ± 1.05^{a}	6.63 ± 0.00^{b}	3.54 ± 0.51^{a}
16:1 (16:1ω9 + 16:1ω7)	0.48 ± 0.00^{ab}	0.34 ± 0.01^{a}	0.67 ± 0.00^{b}	0.59 ± 0.10^{ab}
18:1 (18:1ω9 + 18:1ω7)	24.95 ± 0.00^{c}	21.63 ± 2.19^{a}	24.27 ± 0.00^{bc}	23.10 ± 0.36^{ab}
20:1 (20:1 ω11 + 20:1ω9 + 20:1ω7)	N. d.	0.09 ± 0.00	N. d.	0.06 ± 0.00
Σ MUFA	25.49 ± 0.00^{c}	22.04 ± 1.12^{a}	25.02 ± 0.00^{bc}	23.82 ± 0.33^{b}
16:3ω4	0.09 ± 0.00^{d}	0.06 ± 0.00^{b}	0.05 ± 0.00^{a}	0.07 ± 0.00^{c}
18:2ω6	3.79 ± 0.00^{b}	2.64 ± 0.19^{a}	3.68 ± 0.00^{b}	2.85 ± 0.16^{a}
18:3ω3	0.28 ± 0.00^{b}	0.20 ± 0.01^{a}	0.27 ± 0.00^{b}	0.22 ± 0.01^{a}
20:5ω3	N. d.	N. d.	0.03 ± 0.00	N. d.
Σ PUFA	4.15 ± 0.00^{b}	2.48 ± 0.16^{a}	4.06 ± 0.00^{b}	2.69 ± 0.08^{a}
Σ ω3	0.28 ± 0.00^{b}	0.17 ± 0.01^{a}	0.30 ± 0.00^{b}	0.18 ± 0.01^{a}
Σ ω6	3.79 ± 0.00^{b}	2.27± 0.21^{a}	3.68 ± 0.00^{b}	2.45 ± 0.14^{a}
ω3/ω6	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}

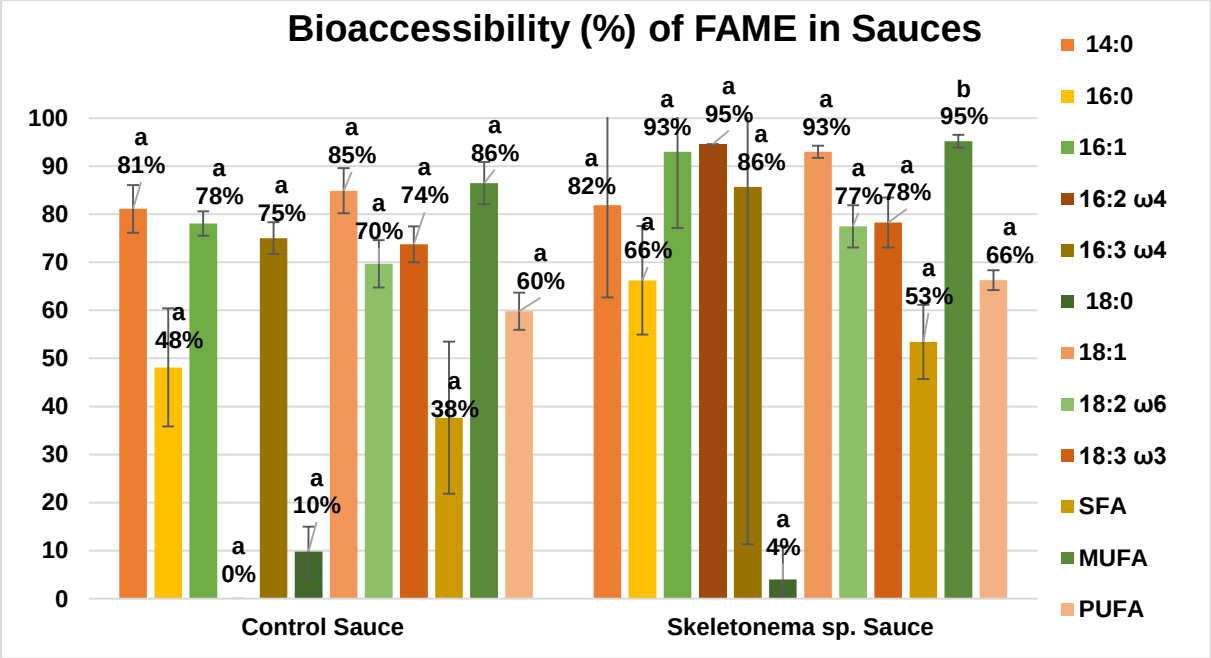

Figure 8 - Bioaccessibility (%) of major FAME's obtained in control and *Skeletonema* sp. sauces. Values are presented as average ± standard deviation. Different lowercase letters represent values statistically different between bioaccessibility percentages ($p < 0.05$) of control and *Skeletonema* sp. sauces.

4.3 Lipid Classes

4.3.1 Algae

The lipid class distribution in the TLC plates for *Skeletonema* sp. using the n-hexane, diethyl ether and formic acid (50:50:2 by volume) elution mixture is illustrated in **Figure 9**. The separation of the lipids is clear with the polar fraction (PL) being very close to the application point and the non-polar fraction migrating away from this point. Although standards were used, there were a few bands that remained unknown, the most relevant being the most distant of the application point, therefore non-polar. The quantification of each separated class is shown of **Table 11**. A non-polar unknown fraction displayed a result of 30.47 ± 0.91 %, being the second major lipid class. The high amount of FFA's (45.99 ± 1.70 %) and the absence of triacylglycerols (TAG) observed in the samples of *Skeletonema* sp. suggest that some hydrolysis may have occurred prior to the assay. Generally, high amounts of FFA are observed in materials that undergo lypolitic degradation. High amounts of FFAs have been reported in diatoms (D'Ippolito et al., 2015). A study by Berge *et al*. (1995) suggested that such findings may be considered as artifacts, indicating unsuitable conditions for lipid extraction, allowing lipase activity development. The use of boiling water prior to lipid extraction eventually proved

to be effective in inhibition of lipase activities and the content of FFA's substantially decreased, which is something to consider in future works.

The bioaccessible fraction of *Skeletonema* sp. is not included in the table, since the lipid content of the diatom was deemed not bioaccessible.

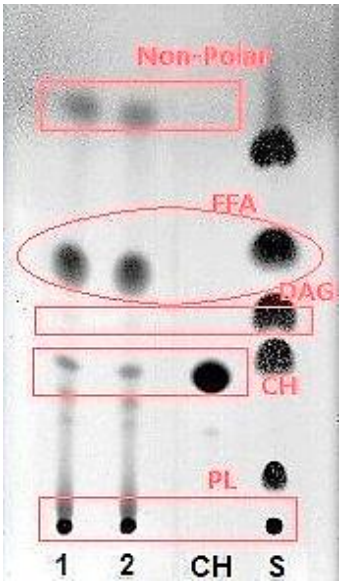

Figure 9 - Lipid class distribution in the TLC plates for *Skeletonema* sp. **1** and **2**), as well as the standards used (CH and S), using an elution mixture of n-hexane, diethyl ether and formic acid (50:50:2 by volume).The polar lipid fraction (PL) stays very close to the application point and the non-polar fraction includes the TAG, FFA, DAG and CH classes. The standards (S) are by order of elution: PL, DAG-1,2, DAG-1,3, FFA and TAG.

Table 11 - Lipid classes separated by TLC in *Skeletonema* sp. Relative percentages of phospholipid (PL), diacylglycerol (DAG), sterol (CH), free fatty acid (FFA), an unknown non-polar fraction and other unknown classes are presented. 1,3-diacylglycerol (DAG-1,3) and 1,2-diacylglycerol (DAG-1,2) are quantified together. The microalgae pigments suffered a co-elution with the polar fraction and are quantified together as well. Values are presented as average ± standard deviation, where N. d. – not detected.

	Skeletonema sp.
PL	8.71 ± 0.97
MAG	N. d.
DAG	N. d.
CH	8.04 ± 0.50
FFA	45.99 ± 1.70
TAG	N. d.
Non-polar	30.47 ± 0.91
Other	7.44 ± 0.67

4.3.2 Food products

The lipid class distribution in the TLC plates of control and with 3 % of *Skeletonema* sp. cookies obtained before and after *in vitro* digestion with an elution mixture of n-hexane, diethyl ether and formic acid (50:50:2 by volume) is illustrated in **Figure 10**. There are very clear differences between initial (prior to digestion) and bioaccessible samples, with a clear density reduction of TAG and, subsequently, increase of FFA density. It is also to be noted the formation of MAGs after the digestion. The quantification of each separated class is shown on **Table 12**.

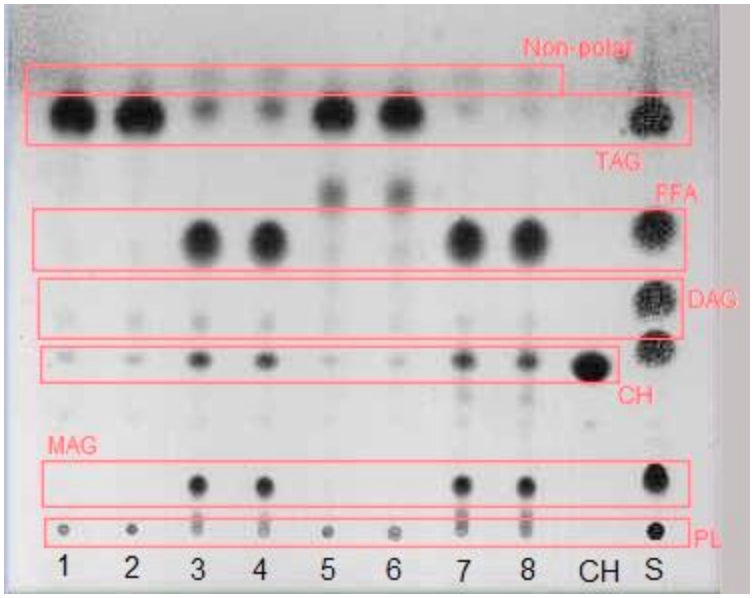

Figure 10 - Lipid class distribution in the TLC plates for Control cookie before (**1** and **2**) and after (**3** and **4**) *in vitro* digestion, as well as *Skeletonema* sp. cookie before (**5** and **6**) and after (**7** and **8**) *in vitro* digestion, using an elution mixture of n-hexane, diethyl ether and formic acid (50:50:2 by volume). The standards used are also displayed (CH and S). The polar lipid fraction (PL) stays very close to the application point and the non-polar fraction includes the TAG, FFA, DAG and CH classes. The standards (S) are by order of elution: PL, DAG-1,2, DAG-1,3, FFA and TAG.

The TLC chromatogram reveals a substantial decrease of TAGs in the bioaccessible fraction of both cookies. Differently, the importance of FFAs displayed a steep increase when compared to the initial sample, reaching 51.44 ± 3.04 % in control cookie and 49.19 ± 0.99 % of total fat in microalgae-containing cookie. Since there was still TAGs found in both bioaccessible cookie samples, it is concluded that the lipid hydrolysis was incomplete, and therefore indicates that the digestion process of the cookies was incomplete. Still, it was detected the formation of MAGs in considerable amounts in both cookies. PL hydrolysis did not occur. In fact, the PL relative content increased in *Skeletonema sp.* cookie bioaccessible samples. Other studies report a total disappearance of TAG class in the bioaccessible fraction, and PL hydrolysis, which was not achieved in this study (Afonso et al., 2017; Costa et al.,

2016). Curiously, the DAG relative content in *Skeletonema* sp. cookie bioaccessible fraction was higher than in the control, followed by a lower content of TAGs, suggesting that the digestion in microalgae cookie was more efficient. However, the limitations of the used *in vitro* method require further testing for confirmation of this observation.

Table 12 - Lipid classes separated by TLC before (Initial) and after *in vitro* digestion (Bio) of Control Cookie and 3 % Skeletonema sp. Cookie. Relative percentages of phospholipid (PL), monoacylglycerol (MAG), diacylglycerol (DAG), sterol (CH), free fatty acid (FFA), triacylglycerol (TAG), an unknown non-polar fraction and other unknown classes are displayed. 1,3-diacylglycerol (DAG-1,3), 1,2-diacylglycerol (DAG-1,2) are quantified together. Values are presented as average ± standard deviation, where N. d. – not detected. Different lowercase letters within a row correspond to statistical differences ($p<0.05$) between initial and bioaccessible fraction of both cookies.

	Control Cookie		**3 % *Skeletonema sp.* Cookie**	
	Initial	**Bio**	**Initial**	**Bio**
PL	2.92 ± 0.18^{a}	4.48 ± 0.51^{ab}	3.32 ± 1.17^{a}	7.11 ± 1.07^{b}
MAG	N. d.^{a}	11.02 ± 0.82^{b}	N. d.^{a}	12.56 ± 1.47^{b}
DAG	9.48 ± 0.63^{b}	7.93 ± 0.81^{b}	5.07 ± 0.46^{a}	12.39 ± 0.24^{c}
CH	6.04 ± 0.63^{a}	6.04 ± 0.63^{a}	3.68 ± 0.81^{a}	3.68 ± 0.81^{a}
FFA	N. d.^{a}	51.44 ± 3.04^{b}	3.57 ± 0.49^{a}	49.19 ± 0.99^{b}
TAG	78.77 ± 0.00^{d}	17.11 ± 1.76^{b}	60.64 ± 1.85^{c}	5.43 ± 1.74^{a}
Non-polar	N. d.^{a}	1.98 ± 0.16^{ab}	N. d.^{a}	7.16 ± 2.54^{b}
Other	2.80. ± 0.18^{a}	N. d.^{a}	23.72 ± 1.26^{b}	2.49 ± 0.85^{a}

The lipid class distribution in the TLC plates of control and with 2 % of *Skeletonema* sp. sauce before and after *in vitro* digestion are illustrated in

Figure 11. An elution mixture of n-hexane, diethyl ether and formic acid (50:50:2 by volume) was used. Once again, there is a very clear density reduction of TAG in initial samples and consequently, an increase of FFA density in bioaccessible fraction. The formation of MAG's after the digestion is also detected. The quantification of each separated class is displayed in **Table 13**.

The analysis of the results shows that the high amount of TAGs present in initial samples was completely hydrolysed in the bioaccessible fraction, resulting in the detection of MAGs and DAGs, that were absent before digestion. The disappearance of TAGs was complete, indicating that a complete digestive process was achieved. As a result of the conversion of TAG to DAG and MAG, the FFAs class had a major increase with results of 51.88 ± 0.48 % in control cookie and 58.70 ± 6.03 % in microalgae cookie. These results are in agreement with other studies that report a high level of lipid hydrolysis with no TAGs detected in the bioaccessible fraction (Afonso et al., 2015; Garcia et al., 2019; Gomes et al., 2019). PL content remained unchanged, therefore hydrolysis did not occur. The bioaccessible fraction of both

sauces did not have many significant differences with only the non-polar unknown class showing a lower result in the *Skeletonema* sp. cookie.

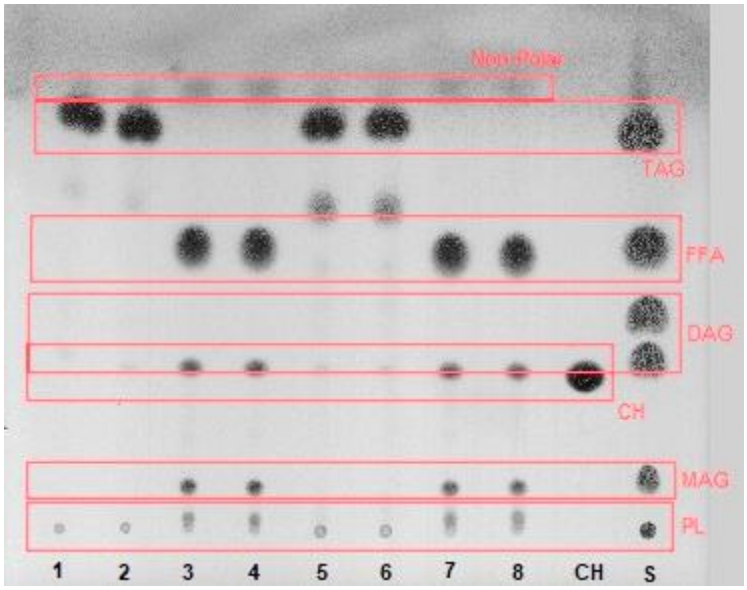

Figure 11 - Lipid class distribution in the TLC plates for control sauce before (**1** and **2**) and after (**3** and **4**) *in vitro* digestion, as well as *Skeletonema* sp. sauce before (**5** and **6**) and after (**7** and **8**) *in vitro* digestion, using an elution mixture of n-hexane, diethyl ether and formic acid (50:50:2 by volume). The standards used are also displayed (CH and S). The polar lipid fraction (PL) stays very close to the application point and the non-polar fraction includes the TAG, FFA, DAG and CH classes. The standards (S) are by order of elution: PL, DAG-1,2, DAG-1,3, FFA and TAG.

Table 13 - Lipid classes separated by TLC before (Initial) and after *in vitro* digestion (Bio) of Control Sauce and 2 % Skeletonema sp. Sauce. Relative percentages of phospholipid (PL), monoacylglycerol (MAG), diacylglycerol (DAG), sterol (CH), free fatty acid (FFA), triacylglycerol (TAG) an unknown non-polar fraction and other unknown classes are displayed. 1,3-diacylglycerol (DAG-1,3), 1,2-diacylglycerol (DAG-1,2) are quantified together. Values are presented as average ± standard deviation, where N. d. – not detected. Different lowercase letters within a row correspond to statistical differences ($p<0.05$) between initial and bioaccessible fraction of both sauces.

	Control Sauce		***2 % Skeletonema sp.* Sauce**	
	Initial	**Bio**	**Initial**	**Bio**
PL	2.85 ± 1.59^{a}	3.71 ± 1.49^{a}	4.16 ± 0.43^{a}	8.26 ± 4.17^{a}
MAG	N. d.^{a}	16.75 ± 3.90^{b}	N. d.^{a}	16.06 ± 1.57^{b}
DAG	N. d.^{a}	0.38 ± 0.54^{a}	N. d.^{a}	3.55 ± 5.03^{a}
CH	4.41 ± 0.57^{a}	3.98 ± 1.18^{a}	5.00 ± 1.32^{a}	4.59 ± 0.74^{a}
FFA	N. d.^{a}	51.88 ± 0.48^{b}	N. d.^{a}	58.70 ± 6.03^{b}
TAG	80.44 ± 0.32^{c}	N. d.^{a}	59.28 ± 3.64^{b}	N. d.^{a}
Non-Polar	N. d.^{a}	23.29 ± 3.19^{c}	N. d.^{a}	8.84 ± 0.84^{b}
Other	12.31 ± 0.70^{b}	N. d.^{a}	31.56 ± 2.75^{d}	N. d.^{a}

4.4 Bioactive Compounds and Antioxidant Activity

4.4.1 Algae

The total polyphenol content and antioxidant activity measured by ABTS, DPPH and FRAP of the aqueous extracts of *Skeletonema* sp. and *Cystoseira abies-marina* is presented in **Table 14**. Regarding the determined total polyphenol content, the seaweed displayed a much higher content than the microalgae, yielding a total of 8.43 ± 0.73 mg GAE/g dw. Studies with other species from the genus *Cystoseira* report a high content of polyphenols, using aqueous extracts, that range between 50.3 and 61.0 mg GAE per g dw (Mhadhebi et al., 2014). The total polyphenolic content of *Skeletonema* sp. was reported to be 2.82-3.68 mg GAE/g dw in various extracts (Franca, 2019), which is in agreement with the values obtained.

The analysis of the antioxidant activity results interweaves three distinct methods, DPPH, ABTS and FRAP. The antioxidant effect of the aqueous extracts by DPPH radical scavenging activity was measured by reducing the stable radical DPPH. The microalgae showed a higher DPPH radical scavenging than the seaweed. ABTS results showed no differences between the two species, both with high values of 41.92 ± 0.17 µmol of Trolox Equivalents per g of dry weight for the microalgae and 43.45 ± 1.23 µmol of Trolox Equivalents per g of dry weight for the seaweed. The results shows clear differences in the total antioxidant capacity as measured by Ferric- Reducing Antioxidant Power (FRAP), since *C. abies-marina* exhibited a much higher value than *Skeletonema* sp., thereby reaching 113.05 ± 8.15 µmol Eq Fe II/g dw. A study with other brown seaweed species (*Petalonia binghamiae* and *Halopteris scoparia*) report a higher ABTS radical scavenging activity with 55-58 µmol Trolox Eq/g dw (Campos et al., 2019)

Due to material and time constraints, the *in vitro* digestion model was only applied to food products with the seaweed *Cystoseira abies-marina*, since it was the species that showed the most promising results regarding bioactive compounds. Consequently, the antioxidant activity and polyphenols content on bioaccessible fraction was only determined in these food products.

Table 14 - Bioactive compounds (total polyphenols) and antioxidant activity measured by DPPH, ABTS and FRAP, in the aqueous extracts of the two algae species, *Skeletonema* sp. and *Cystoseira abies-marina.* Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences ($p<0.05$) between the species.

	Skeletonema sp.	***C. abies-marina*[1]**
Total Polyphenols (mg GAE / g dw)	3.53 ± 0.24[a]	8.43 ± 0.73[b]
DPPH (Eq AA mg / g dw)	0.52 ± 0.00[b]	0.50 ± 0.01[a]
ABTS (µmol Trolox Eq / g dw)	41.92 ± 0.17[a]	43.45 ± 1.23[a]
FRAP (µmol Eq Fe II / g dw)	22.38 ± 0.36[a]	113.05 ± 8.15[b]

[1]Values provided by IPMA

4.4.2 Bioaccessible polyphenols and bioactivities of *C. abies-marina*

The *in vitro* digestion model was performed with the seaweed *Cystoseira abies-marina* and the total polyphenolic content as well as the antioxidant activity (measured by DPPH and FRAP) were calculated in the bioaccessible fraction as shown in **Table 15**. The high content of polyphenols found in *C. abies-marina* was proven to be totally bioaccessible with similar results being attained in initial and bioaccessible samples. The DPPH radical scavenging activity varied a lot between the initial and bioaccessible samples, with the digested samples having a much lower value than the initial sample. Possibly the components of the digestive juices had some radical scavenging ability or other interferents that affected the assay (note that the absorbance of the bioaccessible blank was subtracted). The FRAP assay in the bioaccessible samples produced a relatively high value, even if lower than the initial sample, reaching values of 86.61 ± 3.52 µmol Eq Fe II per g of dry weight. The ABTS assay was not performed due to material and time constraints.

The bioaccessibility percentage of the performed assay is shown on **Figure 12**. Although total phenolic content and FRAP assay showed high bioaccessibility, DPPH assay had a bioaccessibility of 4 % in this seaweed. Studies of bioaccessibility of compounds with antioxidant activity measured by FRAP and DPPH are still scarce. Most studies are on different food matrix and show a low bioaccessibility of total phenolic content in digested samples, with values that go as low as 16 % (Figueroa et al., 2016; Pérez-Vicente et al., 2002; Tagliazucchi et al., 2010). Differently, a study with fruit beverages showed similar results to this work with 96 % of bioaccessibility of polyphenols (Cilla et al., 2011). The antioxidant capacity after the simulated *in vitro* digestion measured by DPPH was much lower that the reported by a study

with walnuts, averaging 31 % (Figueroa et al., 2016). Regarding antioxidant activity measured by FRAP, Bouayed et al. (2011) reached a similar result with 70 % of recovery from fresh apple matrix.

Table 15 - Bioactive compounds (total polyphenols) and antioxidant activity measured by DPPH and FRAP before (Initial) and after in vitro digestion (Bio) of *Cystoseira abies-marina.* Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences ($p<0.05$) between initial and bioaccessible fraction of the seaweed.

	***C. abies-marina*[1]**	
	Initial	**Bio**
Total Polyphenols (mg GAE / g dw)	8.43 ± 0.73[a]	8.87 ± 1.34[a]
DPPH (Eq AA mg / g dw)	0.50 ± 0.01[b]	0.02 ± 0.01[a]
FRAP (µmol Eq Fe II / g dw)	113.05 ± 8.15[b]	86.61 ± 3.52[a]

[1]Values provided by IPMA

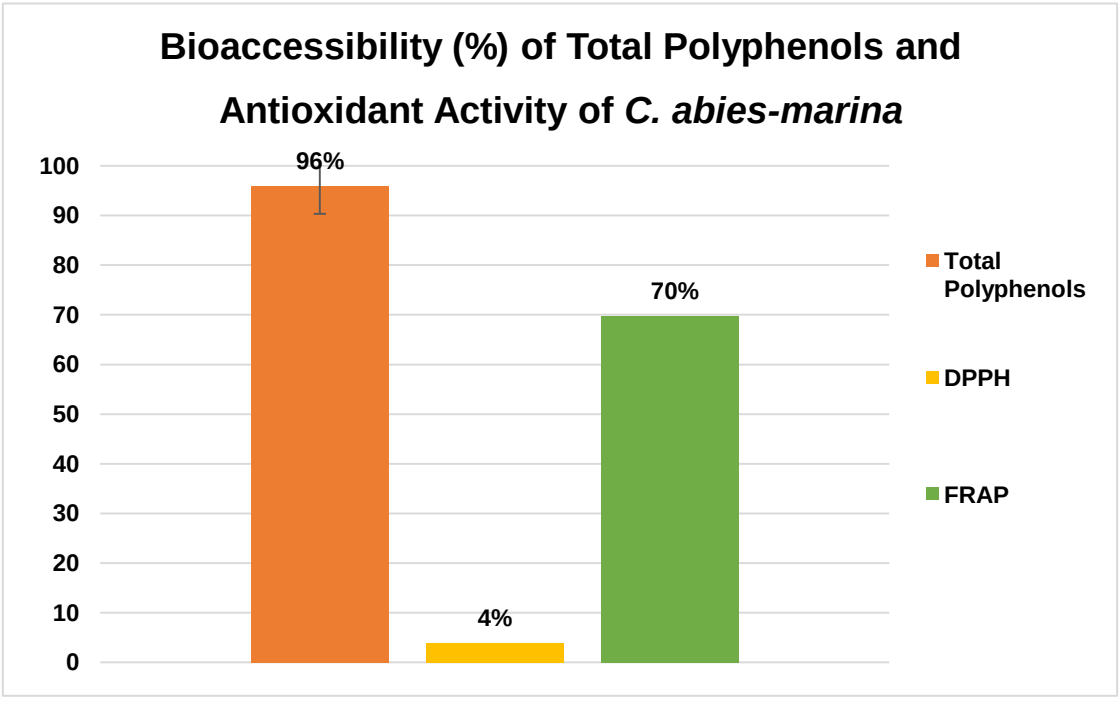

Figure 12 - Bioaccessibility (%) of bioactive compounds (total polyphenols) and antioxidant activity measured by DPPH and FRAP of *Cystoseira abies-marina*. Values are presented as average ± standard deviation.

4.4.3 Food Products

The total polyphenol content and antioxidant activity, measured by DPPH, FRAP and ABTS, of the food products are displayed in **Table 16**. The microalgae sauce and the seaweed cookie had the highest total polyphenol content, thereby yielding a total of 1.12 ± 0.04 mg GAE per g dw and 0.93 ± 0.06 mg GAE per g dw, respectively. It was expected a higher amount of polyphenols in the seaweed sauce since *C. abies-marina* had a higher polyphenol content than the microalgae. A better homogenization and a larger quantity of seaweed would be required to avoid such result. In any case, differently from *Skeletonema* sp. addition, the incorporation of *C. abies-marina* in the cookies was effective in increasing polyphenol content with respect to the control. Sesame seeds were an ingredient used in the cookie, although not in high amount. This seed have a high content of polyphenols that range between 3.71 – 7.86 mg GAE/g dw (Lin et al., 2017), that may be responsible for the polyphenolic content of the cookies. Olive oil is known for having high polyphenolic content (138-278 mg GAE/kg oil) (Negro et al., 2019) and white onion has really high total polyphenolic content with 26.44 mg GAE/ g dw. Both are ingredients of the sauces, explaining why sauces polyphenolic content is higher than the cookies.

Regarding the DPPH radical scavenging activity, the products with the seaweed showed the highest antioxidant activity. ABTS results were strongly different comparing cookies and sauce, the latter having far higher inhibition. The *C. abies-marina* cookie revealed the highest activity of all the cookies, despite the fact the microalgae and the seaweed had similar antioxidant activity measured by ABTS (**Table 14**). This suggests that the high temperatures experienced by the baking of the cookies may have affected the antioxidant activity of *Skeletonema* sp. The opposite was verified in the sauces with *Skeletonema* sp. sauce having the highest ABTS value, thereby reaching a total of 35.83 ± 0.22 µmol Trolox Eq / g dw. Again the *C. abies-marina* sauce showed less activity than the expected. Concerning the FRAP assay, the sauces revealed a better antioxidant capacity with the seaweed sauce displaying the highest value with 13.07 ± 0.11 µmol Eq Fe II per g of dry weight. The microalgae sauce did not show significant differences with respect to the control sauce, which is in agreement with the fact that the seaweed had a much higher FRAP value than *Skeletonema* sp. The cookies had a similar result, with *Cystoseira abies-marina* cookie having the best result of the 3 cookies and *Skeletonema* sp. cookie not showing a significant different antioxidant activity when compared with the control cookie.

Overall, the sauces revealed a higher antioxidant activity than the cookies, being the ABTS assay the most evident. The fact that the sauces were not exposed to high temperatures and the ingredients were simply mixed, may have been influential in preventing the oxidation of important bioactive compounds. Studies of antioxidant properties of onion, one of sauces main

ingredient, report high FRAP values that range between 91-187 μmol Eq Fe II / g dw , depending on the cultivar and also really high ABTS activity ranging between 126-295 μmol Trolox Eq / g dw (Burri et al., 2017). This explains the higher antioxidant activity values found on sauces in comparison to the cookies, since olive oil and onion are sauces ingredients that possess a good amount of bioactive compounds.

Table 16 - Bioactive compounds (total polyphenol content) and antioxidant activity measured by DPPH, ABTS and FRAP, obtained in the food products. Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences ($p<0.05$) between the food products.

	Control Cookie	**3% *C. abies-marina* Cookie**	**3% *Skeletonema sp.* Cookie**	**Control Sauce**	**2% *C. abies-marina* Sauce**	**2% *Skeletonema sp.* Sauce**
Total Polyphenols (mg GAE / g dw)	0.32 ± 0.03^{a}	0.93 ± 0.06^{c}	0.41 ± 0.03^{a}	0.71 ± 0.01^{b}	0.77 ± 0.02^{b}	1.12 ± 0.04^{d}
DPPH (Eq AA mg / g dw)	0.20 ± 0.02^{a}	0.41 ± 0.01^{c}	0.27 ± 0.01^{b}	0.38 ± 0.01^{c}	0.47 ± 0.01^{d}	0.41 ± 0.01^{c}
ABTS (µmol Trolox Eq / g dw)	2.33 ± 0.62^{a}	8.39 ± 0.91^{c}	4.61 ± 0.62^{b}	25.73 ± 0.49^{d}	28.44 ± 0.87^{e}	35.83 ± 0.22^{f}
FRAP (µmol Eq Fe II / g dw)	4.02 ± 0.82^{a}	7.56 ± 0.30^{b}	3.92 ± 0.08^{a}	11.27 ± 0.09^{c}	13.07 ± 0.11^{d}	11.52 ± 0.35^{c}

4.4.4 Bioaccessible polyphenols and bioactivities of food products

The *in vitro* digestion model was applies to the control product and the product incorporating the seaweed *Cystoseira abies-marina* in order to assess the total polyphenolic content as well as the antioxidant activity (measured by DPPH and FRAP) in the bioaccessible fraction, being the results per edible part displayed in **Table 17**.The polyphenolic content showed some unexpected results with the bioaccessible fraction exhibiting higher polyphenolic content than the initial samples. Possibly, there may have been some interferences of the digestive juices in the determination of the phenolic content or the digestion generated additional compounds more accessible to be measured by this assay. Nevertheless, it is to be noted that the bioaccessible fraction of both cookies and both sauces had similar polyphenolic content, with the cookies showing a higher value. The DPPH radical scavenging activity decreased in the bioaccessible fraction of both products with respect to the initial activity. The cookies and sauces did not show a significant difference in antioxidant activity in bioaccessible samples. The FRAP assay in the bioaccessible samples produced very different results in the two food products. In the cookies, the ability to reduce Fe^{3+} was not detected in the digested samples, possibly due to some interferences of the components present in the digestive juices and the cookies. The sauces showed a similar activity before and after digestion, enabling the conclusion that all bioactive compounds able to reduce Fe^{3+} were rendered bioaccessible. The ABTS assay was not performed due to material and time constraints.

The bioaccessible percentages of the bioactive compounds and antioxidants activity obtained are presented in **Figure 13**. Similarly to what was observed in the bioaccessible algae fraction, total phenolic content and FRAP assay presented a high bioaccessible percentage in contrast to the antioxidant activity measured by DPPH. Although the high bioaccessibility rates of phenolic compounds and antioxidant activity attained by FRAP were higher than the reported in other studies with different food matrix, the DPPH bioaccessibility values were lower than the reported (Figueroa et al., 2016; Pérez-Vicente et al., 2002; Tagliazucchi et al., 2010).

Table 17 - Bioactive compounds (total polyphenols) and antioxidant activity measured by DPPH, ABTS and FRAP, obtained before (Initial) and after *in vitro* digestion (Bio) in the control food products and with *Cystoseira abies-marina* Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences ($p<0.05$) between initial (prior to digestion) fraction of the products. Different lowercase letters within a row correspond to statistical differences ($p<0.05$) between bioaccessible fraction of the products.

	Control Cookie		**3% *C. abies-marina* Cookie**		**Control Sauce**		**2% *C. abies-marina* Sauce**	
	Initial	**Bio**	**Initial**	**Bio**	**Initial**	**Bio**	**Initial**	**Bio**
Total Polyphenols (mg GAE / g)	0.32 ± 0.03^{b}	0.81 ± 0.13^{B}	0.89 ± 0.05^{c}	0.62 ± 0.16^{B}	0.10 ± 0.00^{a}	0.39 ± 0.06^{A}	0.11 ± 0.00^{a}	0.38 ± 0.05^{A}
DPPH (Eq AA mg / g)	0.20 ± 0.02^{c}	0.05 ± 0.00^{B}	0.39 ± 0.01^{d}	0.05 ± 0.00^{B}	0.05 ± 0.00^{a}	0.01 ± 0.00^{A}	0.07 ± 0.00^{b}	0.01 ± 0.01^{A}
FRAP (µmol Eq Fe II / g)	3.96 ± 0.81^{c}	N. d.^{A}	7.27 ± 0.29^{d}	N. d.^{A}	1.61 ± 0.01^{a}	1.83 ± 0.48^{B}	1.93 ± 0.02^{b}	2.24 ± 0.77^{B}

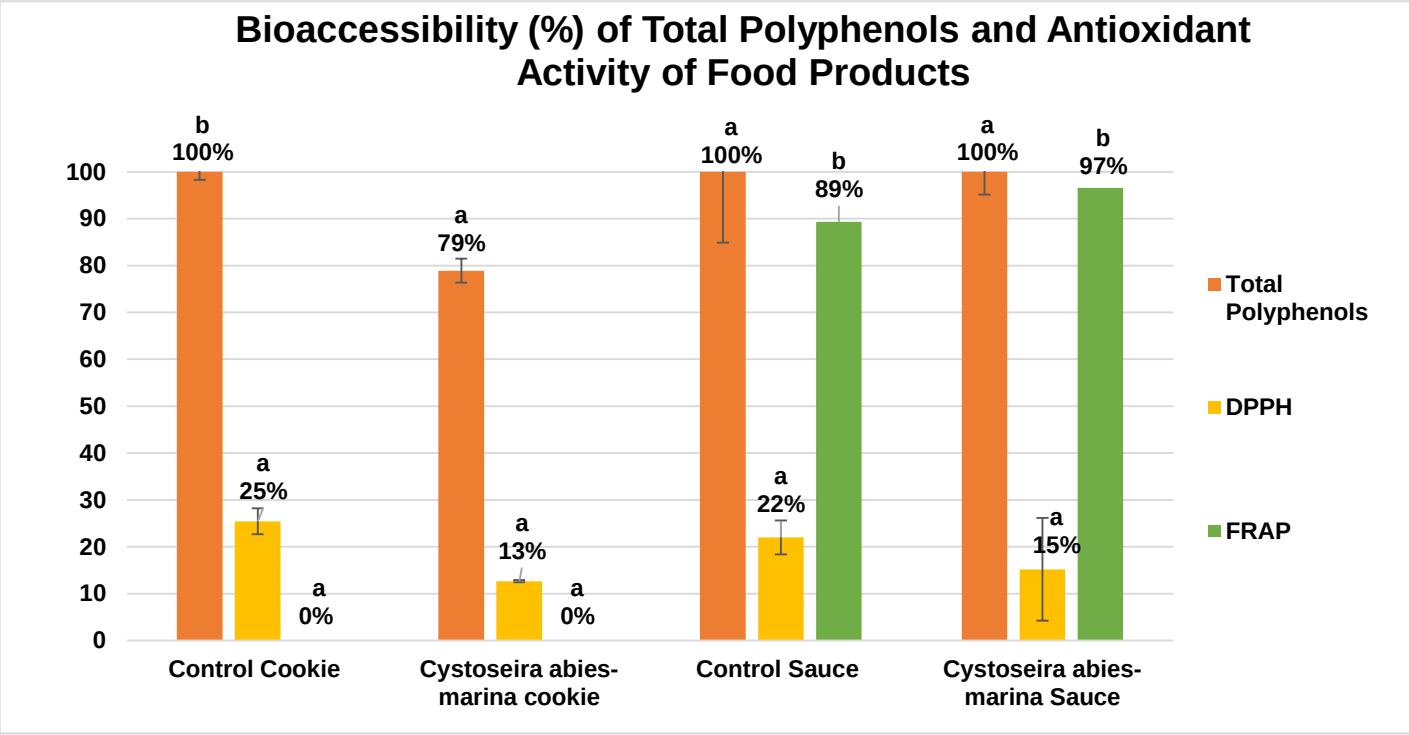

Figure 13 - Bioaccessibility (%) of bioactive compounds (total polyphenols) and antioxidant activity measured by DPPH and FRAP of control and with *Cystoseira abies-marina* food products. Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences ($p<0.05$) between bioaccessible fraction of the products.

4.5 Mineral composition

4.5.1 Algae

The elemental composition of the microalgae *Skeletonema* sp. and macroalgae *Cystoseira abies-marina* is presented in **Table 18**. Concerning essential elements, in the diatom, calcium was found in the highest concentration 46.82 ± 3.45 g/kg dw, followed by sodium with 21.65 ± 0.76 g/kg dw. In *C. abies-marina* potassium was the most abundant mineral with 46.22 ± 1.82 g/kg dw, followed by sodium with 31.51 ± 0.41 g/kg dw. These high values were also reported in other *Cystoseira* species (Vizetto-Duarte et al., 2016). *Skeletonema* sp. displayed a higher content of iron, calcium and phosphorus than the macroalgae. In contrast, *Cystoseira* presented a higher amount of potassium and sodium. Seaweeds typically contain high concentrations of calcium, sodium, potassium and magnesium (Taboada et al., 2010). Moreover, it is reported that brown seaweeds seem to have higher accumulation capacity of some contaminants such as As and Pb, than red and green macroalgae (Squadrone et al., 2018). The Na/K ratio found in *Cystoseira* is below 1.0, which is interesting from a nutritional point of view as diets with a high Na/K ratio have been associated with the incidence of hypertension (Taboada et al., 2010). Zinc and magnesium were found in similar concentrations in both species. The calcium, sodium and magnesium content found in *Skeletonema* was higher than in the commercial microalgae *Spirulina* and *Chlorella*, in contrast to the much lower iron content (Rzymski et al., 2018). Kumar and Prabu (2015) studied the trace minerals found in *Skeletonema costatum*, with calcium , sodium and potassium being the most abundant, in agreement with these results. It is important to note that, for microalgae produced in bioreactors, its mineral content is largely influenced by the composition of the culture medium, since algae have accumulative properties. Regarding contaminant elements, it is noted the high amount of arsenic found in *C. abies-marina* (340.07 ± 0.18 mg/kg dw), which was not expected. A speciation study would be required to assess the level of toxicity of the arsenic found. The lead and copper concentration was similar in both species, lead being found in a low amount. Squadrone et al. (2018) reported a much lower arsenic content in *Cystoseira* species with 20 ± 0.78 mg/kg dw. On the other hand, lead and copper were found in a higher concentration. In addition, it is worth mentioning that the contaminant and essential elements concentration in algae is controlled by the environmental factors specific to a given location that influence the elemental content in water (Afonso et al., 2018). With this in mind, the differences in elemental content between algae of the same species or same genus are explained by the location and environment of the algae.

Table 18 - Mineral composition obtained in the microalgae *Skeletonema sp.* and macroalgae *Cystoseira abies-marina.* Concentration of lead (Pb), copper (Cu), Iron (Fe), Zinc (Zn), Arsenic (As), Potassium (K), Calcium (Ca), Magnesium (Mg), Sodium (Na) and Phosphorus (P) are displayed. Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences ($p<0.05$) between the algae.

	Skeletonema sp.	***C. abies-marina*[1]**
Pb (mg/kg dw)	0.26 ± 0.05[a]	0.28 ± 0.21[a]
Cu (mg/kg dw)	11.47 ± 5.12[a]	6.43 ± 0.24[a]
Fe (mg/kg dw)	2.51 ± 0.51[b]	0.04 ± 0.00[a]
Zn (mg/kg dw)	78.81 ± 37.16[a]	3.24 ± 0.39[a]
As (mg/kg dw)	4.51 ± 1.24[a]	340.07 ± 0.18[b]
K (g/kg dw)	14.19 ± 0.06[a]	46.22 ± 1.82[b]
Ca (g/kg dw)	46.82 ± 3.45[b]	11.26 ± 0.40[a]
Mg (g/kg dw)	5.42 ± 0.30[a]	6.02 ± 0.19[a]
Na (g/kg dw)	21.65 ± 0.76[a]	31.51 ± 0.41[b]
P (g/kg dw)	8.05 ± 0.23[b]	0.35 ± 0.01[a]

[1] Values provided by IPMA

4.5.2 Bioaccessible minerals of *C. abies-marina*

The *in vitro* digestion model to asses mineral bioaccessibility was only performed in *C. abies-marina* and in the food products with this brown seaweed, due to lack of resources. The results for the mineral elements before and after *in vitro* digestion of *C. abies-marina* are presented in the **Table 19**. It can be verified that there is a significant reduction of some elements concentration after *in vitro* digestion. Magnesium, arsenic, iron and calcium revealed similar concentrations in initial and bioaccessible fraction. The remained elements showed that the human digestion might not be able to fully release the compounds from its matrix. The bioaccessibility percentages of essential elements are shown in **Figure 14**. Arsenic, potassium and magnesium were the elements that presented the highest bioaccessibility percentage. In contrast, copper, zinc and phosphorus showed to be not bioaccessible in this seaweed. The low percentage of bioaccessibility of zinc and high bioaccessibility of arsenic and lead was also shown in studies with green seaweed (Afonso et al., 2018). Boato et al. (2002) observed that fruit juices high in polyphenols content limited bioavailability of iron by forming iron–polyphenol complexes, preventing absorption by the cells. Knowing that *C. abies-marina* has a high polyphenolic content, the low bioaccessibility of iron observed may be due to the formation of such complexes. To explain the low bioaccessibility of other minerals in this seaweed, Taboada et al. (2010) suggested that binding of certain minerals to the

polysaccharides present in seaweeds may limit its absorption. Also, this species may contain anti-nutritional factors, such as phytate and some of its degradation products, that are well-known inhibitors of absorption of essential dietary minerals in legumes, in particular iron and Zn (Sandberg, 2002), explaining their low bioaccessibility. In fact, Ardiansyah et al. (2018) measured the content of phytic acid present in *Sargassum* sp. powder, which is a brown seaweed as well, and obtained a result of 22.35 ± 0.39 mg/g.

Table 19 - Mineral composition obtained in the macroalgae *Cystoseira abies-marina* before (Initial) and after *in vitro* digestion (Bio). Concentration of lead (Pb), copper (Cu), iron (Fe), zinc (Zn), arsenic (As), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na) and phosphorus (P) are displayed. Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences ($p<0.05$) between the initial and bioaccessible fraction.

	***C. abies-marina*[1]**	
	Initial	**Bio**
Pb (µg/g dw)	0.61	0.37
Cu (µg/g dw)	2.25 ± 0.08[b]	N. d.[a]
Fe (µg/g dw)	41.16 ± 13.14[a]	11.29[a]
Zn (µg/g dw)	14.38 ± 2.77[b]	N. d.[a]
As (mg/g dw)	0.26 ± 0.02[a]	0.21 ± 0.02[a]
K (mg/g dw)	51.10 ± 0.35[b]	37.05 ± 1.99[a]
Ca (mg/g dw)	8.77 ± 2.15[a]	1.89 ± 1.22[a]
Mg (mg/g dw)	5.60 ± 0.75[a]	4.26 ± 0.58[a]
Na (mg/g dw)	44.59 ± 4.62[b]	8.01 ± 0.23[a]
P (mg/g dw)	0.96 ± 0.08[b]	N. d.[a]

[1] Values provided by IPMA

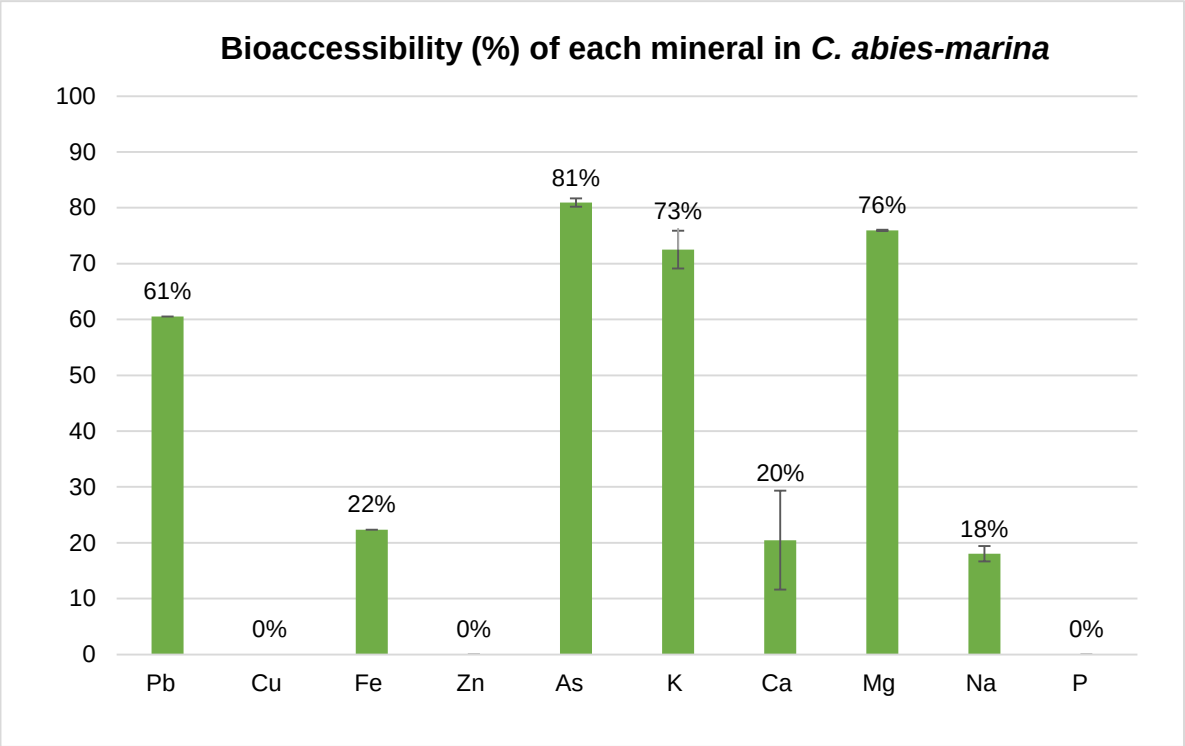

Figure 14 - Bioaccessibility percentages (%) of each mineral obtained in the macroalgae *Cystoseira abies-marina.* Bioaccessible percentage of lead (Pb), copper (Cu), iron (Fe), zinc (Zn), arsenic (As), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na) and phosphorus (P) are displayed. Values are presented as average ± standard deviation

4.5.3 Food products

The composition of each mineral in the food products was determined and is presented in **Table 20**. Regarding contaminant elements, lead and copper presented a similar content in all food products analysed, suggesting that the amount of algal biomass used in the products was insufficient to produce any change. This was not the case for arsenic, which showed a significant higher amount of arsenic in food products with *Cystoseira,* which is consistent with the high amount of arsenic in this seaweed. Concerning essential elements, sodium was the element found in highest concentration in both cookies and sauces, which is expected since salt was one of the ingredients used in the products. Sauces presented highest concentration of elements zinc, potassium, calcium, sodium and phosphorus compared to cookies. Onion and natural yogurt, two major ingredients in the sauces, contain higher values of potassium, calcium and phosphorus, than the major ingredients used in the preparation of cookies, margarine and flour, which explains the differences (Bello et al., 2013; INSA, 2019).

Table 20 - Mineral composition obtained in control and with macroalgae *Cystoseira abies-marina* food products. Concentration of lead (Pb), copper (Cu), iron (Fe), zinc (Zn), arsenic (As), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na) and phosphorus (P) are displayed. Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences ($p<0.05$) between the cookies and the sauces.

	Control Cookie	3% *C. abies-marina* Cookie	Control Sauce	2% *C. abies-marina* Sauce
Pb (mg/kg dw)	0.26 ± 0.02^{a}	0.40 ± 0.15^{a}	0.35 ± 0.05^{a}	0.21 ± 0.09^{a}
Cu (mg/kg dw)	7.07 ± 1.32^{a}	5.77 ± 1.11^{a}	8.85 ± 0.07^{a}	6.82 ± 0.58^{a}
Fe (mg/kg dw)	16.68 ± 0.08^{a}	20.91 ± 3.03^{ab}	25.68 ± 1.80^{b}	21.15 ± 1.55^{ab}
Zn (mg/kg dw)	7.68 ± 1.30^{a}	7.59 ± 0.40^{a}	20.88 ± 0.18^{c}	16.15 ± 1.41^{b}
As (mg/kg dw)	3.68 ± 0.23^{a}	15.93 ± 1.33^{b}	4.82 ± 0.65^{a}	40.36 ± 2.70^{c}
K (g/kg dw)	1.41 ± 0.26^{a}	2.29 ± 0.07^{a}	9.94 ± 0.89^{b}	12.91 ± 1.68^{b}
Ca (g/kg dw)	0.92 ± 0.04^{a}	1.19 ± 0.17^{a}	3.70 ± 0.25^{b}	4.25 ± 0.02^{b}
Mg (g/kg dw)	0.21 ± 0.01^{a}	0.38 ± 0.06^{ab}	0.68 ± 0.04^{bc}	1.00 ± 0.14^{c}
Na (g/kg dw)	2.78 ± 0.25^{a}	3.18 ± 0.02^{a}	15.29 ± 1.34^{b}	15.22 ± 2.27^{b}
P (g/kg dw)	1.62 ± 0.03^{a}	1.49 ± 0.20^{a}	3.98 ± 0.10^{b}	3.17 ± 0.36^{b}

4.5.4 Bioaccessible minerals of food products

The *in vitro* digestion model was performed on fresh food products to assess the bioaccessible mineral fraction of the food products (control and with *C. abies-marina*), with the results presented in **Table 21**. The percentage of bioaccessibility of the compounds was calculated and is displayed in **Figure 15.** The results show that lead (Pb), copper (Cu), iron (Fe) and zinc (Zn) were not detected in the bioaccessible fraction of the sauces, being zinc the only mineral that showed a significant decrease in concentration in the bioaccessible fraction compared to the initial samples in both products. Essential elements like sodium, magnesium, potassium, calcium and phosphorus showed similar results in initial and bioaccessible fractions of both products, resulting in high bioaccessibility percentages, some reaching 100 %. Regarding contaminants, despite the reduction of lead and copper content in most products, arsenic showed a similar content in initial and bioaccessible fraction. Zinc and lead had the lowest bioaccessibility percentages.

High arsenic bioaccessibility and low Fe and Pb was observed in a study with molluscs in agreement with the current study's results, however the same study showed high

bioaccessibility of copper (He and Wang, 2013). Although zinc bioaccessibility varied a lot between studies, in green seaweed *Chaetomorpha linum* and *Ulva intestinalis* similar results with no bioaccessible zinc were attained (Afonso et al., 2018). High magnesium bioaccessibility was observed but in a 75 % percentage in a study with whole grain tea biscuits (Vitali et al., 2008). Calcium bioaccessibility was reported low in various studies with legumes and biscuits (Sahuquillo et al., 2003; Vitali et al., 2008), ranging from 22 to 55 %, which was not observed in this study. High potassium bioaccessibility was reported in a study with *Sarcocornia ambigua* samples ranging between 73-80 %, close to the bioaccessibility percentages attained in this study (Bertin et al., 2016).

Table 21 - Mineral composition obtained before (Initial) and after (Bio) *in* vitro digestion of control and with macroalgae *Cystoseira abies-marina* food products. Concentration of lead (Pb), copper (Cu), iron (Fe), zinc (Zn), arsenic (As), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na) and phosphorus (P) per g of fresh food product are displayed. Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences ($p<0.05$) between the initial and bioaccessible fraction.

	Control Cookie		**3% *C. abies-marina* Cookie**		**Control Sauce**		**2% *C. abies-marina* Sauce**	
	Initial	**Bio**	**Initial**	**Bio**	**Initial**	**Bio**	**Initial**	**Bio**
Pb (µg/g)	0.11^{b}	N. d.^{a}	0.06^{ab}	N. d.^{a}	0.03 ± 0.02^{a}	N. d.^{a}	0.02^{a}	N. d.^{a}
Cu (µg/g)	7.19 ± 0.27^{d}	4.73 ± 0.92^{bc}	4.94 ± 0.44^{c}	3.29 ± 0.08^{b}	0.49 ± 0.10^{a}	N. d.^{a}	0.81 ± 0.21^{a}	N. d.^{a}
Fe (µg/g)	10.24 ± 3.30^{c}	4.70 ± 0.74^{abc}	8.79 ± 2.78^{bc}	7.70^{abc}	0.83 ± 0.11^{ab}	N. d.^{a}	4.01 ± 2.68^{abc}	N. d.^{a}
Zn (µg/g)	7.76 ± 0.19^{e}	0.41^{ab}	5.59 ± 0.02^{d}	N. d.^{a}	0.80 ± 0.04^{b}	N. d.^{a}	3.25 ± 0.19^{c}	N. d.^{a}
As (µg/g)	4.95 ± 0.09^{ab}	4.61 ± 0.12^{ab}	11.97 ± 1.40^{c}	10.68 ± 1.51^{c}	1.48 ± 1.01^{ab}	1.31 ± 1.06^{a}	5.02 ± 0.45^{b}	4.11 ± 0.50^{ab}
K (mg/g)	0.81^{abc}	0.52^{ab}	2.04 ± 0.31^{c}	1.45 ± 0.14^{bc}	0.41 ± 0.09^{a}	0.32 ± 0.07^{a}	0.99^{abc}	0.96^{abc}
Ca (mg/g)	0.28 ± 0.03^{a}	0.28 ± 0.03^{a}	0.19^{a}	0.19^{a}	0.39 ± 0.33^{a}	0.39 ± 0.33^{a}	0.32^{a}	0.32^{a}
Mg (mg/g)	0.15 ± 0.03^{ab}	0.15 ± 0.03^{ab}	0.36 ± 0.13^{b}	0.25 ± 0.02^{ab}	0.11 ± 0.04^{a}	0.11 ± 0.04^{a}	0.15 ± 0.07^{ab}	0.15 ± 0.07^{ab}
Na (mg/g)	5.74 ± 3.19^{a}	5.61 ± 3.11^{a}	8.65 ± 3.24^{a}	7.27 ± 1.35^{a}	2.67^{a}	2.64^{a}	1.69^{a}	1.67^{a}
P (mg/g)	1.39 ± 0.06^{bc}	1.39 ± 0.06^{bc}	1.55 ± 0.41^{c}	1.22 ± 0.05^{bc}	0.55 ± 0.25^{ab}	0.55 ± 0.25^{ab}	0.21 ± 0.19^{a}	0.21 ± 0.19^{a}

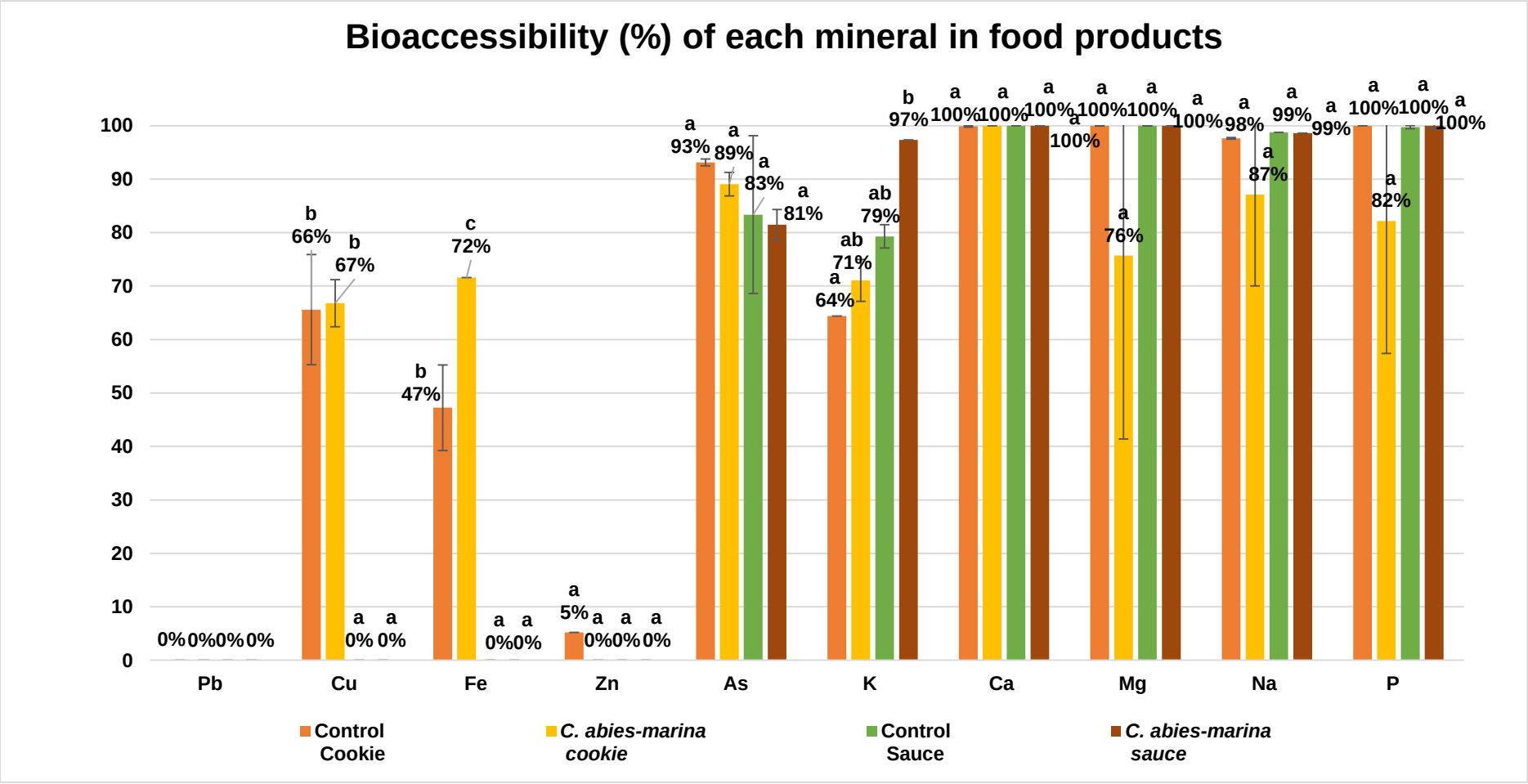

Figure 15 - Bioaccessibility percentages (%) of each mineral obtained in the control and with *Cystoseira abies-marina* food products. Bioaccessible percentage of lead (Pb), copper (Cu), iron (Fe), zinc (Zn), arsenic (As), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na) and phosphorus (P) are displayed. Values are presented as average ± standard deviation. Different lowercase letters correspond to statistical differences ($p<0.05$) between the bioaccessibility of each mineral of the food products.

5. Conclusions and Future Perspectives

Over the last decade, algae has been marketed as a nutritional and healthy food, with enormous potential to revolutionize food industry. In this study, the nutritional and bioactive properties of the microalgae *Skeletonema* sp. and brown seaweed *Cystoseira abies-marina* were evaluated in order to understand their potential to be incorporated in food products. The results of their nutritional value and antioxidant activity were in agreement with previous studies, revealing interesting properties. Two different food products with both algae incorporated were prepared and analysed, revealing significant improvement in polyphenols content and antioxidant activity. The results of the estimation of bioaccessibility indicate that the majority of the polyphenols and antioxidants measured by FRAP can resist to the digestion conditions, being rendered available for absorption by the intestine cells.

Regarding the nutritional value of both food products, the cookies presented a higher PUFA content with 40 %, with a bioaccessibility of 60 %. However sauces presented a low saturated fatty acid content with low bioaccessibility, which is more attractive to health-conscious consumers. Minerals like calcium, potassium, phosphorus, and magnesium showed high bioaccessibility in both food products. From the contaminants analyses, it can be stated that *Cystoseira* displayed high levels of arsenic that resulted in a significant increase of this contaminant in the food products containing this seaweed. Moreover, it is to note its high bioaccessibility values, which may be an additional concern.

Due to time constrains, some analysis were not completed and would be interesting to perform in the future, such as measuring the non-digested fraction of the *in vitro* model to determine more accurate bioactivities and total phenol content, applying the *in vitro* digestion model to food products with *Skeletonema,* and carrying out an anti-inflammatory activity assay, and performing a stability study of the food products. Additionally, it would be relevant to assess the iodine content in both species since algae are known to be excellent sources of iodine, and perform a speciation study of the arsenic to determine what form (organic or inorganic) is present and, consequently, what risks pose to human health.

In future works, it would be important to carry out a sensory analysis of the food products prepared, in order to determine consumer acceptance and preference. The seaweed *Cystoseira abies-marina* had a more subtle taste than *Skeletonema*, resulting in food products with similar taste to the control. The sugar content of the cookies resulted in a higher kilocalorie energy value compared to the sauces. An improvement to the recipe could aim to reduce the sugar content, thereby making cookies a more healthy food.

Although this work gave much information regarding composition, bioactivities, and bioaccessibility of different nutrients, in future studies, it would be of interest to identify the specific antioxidant compounds that were bioaccessible, to speciate arsenic in *C. abies-marina*

(for a better assessment of the risk associated to this seaweed consumption), and to evaluate how seasons affect the production of antioxidants and lipids (not only in wild seaweed, but also in microalgae cultivated outdoors), since they are deemed to vary depending on the climatic conditions.

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7. Annexes

7.1 List of ingredients and preparation of yogurt sauces

The ingredients and quantities used for the preparation of the yogurt sauce are described in **Table 22**. In order to prepare to yogurt sauce, firstly the onion and garlic were minced together, then the rest of the ingredients were added and minced. The resulting mixture was weighed and divided in 3 equal portions, where one portion was used as control sauce with nothing added, one portion was weighed and added 2 % *of Skeletonema* sp., and the final portion was weighed and added 2 % of *Cystoseira abies-marina*.

Table 22 - List of ingredients, and its respective mass, used to prepare the yogurt sauce. This list refers to the control sauce preparation, which was divided in 3 equal portions before adding the algae.

SAUCE INGREDIENTS	Mass (g)
Skinny natural yogurt	375.0
Lemon juice	29.9
Olive oil	27.1
Garlic	15.4
Onion	216.6
Black Pepper	0.5
Salt	5.0
Peppermint	1.1

7.2 List of ingredients and preparation of cookies

For the preparation of the cookies, the ingredients and respective masses used are presented in **Table 23**. The recipe for the cookies encompassed an initial mixing of sugar with margarine until they formed a homogenous dough. Then the flour, cornflour, baking soda, roasted sesame seeds and salt were incorporated in the mixture. The total mass was weighted and divided in 3 equal parts where one part was used as control cookie with nothing added, one part was weighed and had 3 % *of* incorporated *Skeletonema* sp., and the final part was weighed and 3 % of *Cystoseira abies-marina* was incorporated. The 3 resulting doughs were left resting for 30 minutes at room temperature and then divided into small balls. A thin layer of egg wash was applied on top of the cookies and then baked for 12 minutes at 180 ºC.

Table 23 - List of ingredients, and its respective mass, used to prepare the cookies. This list refers to the control cookie preparation, which was divided in 3 equal portions before adding the algae.

COOKIES INGREDIENTS	Mass (g)
Sesame seeds	40.2
Margarine	201.9
Sugar	119.9
Flour	160.0
Cornflour	120.4
Baking Soda	0.8
Salt	2.7
Egg	51.1

7.3 Poster presented in the 49th WEFTA Conference

Some results presented in this work regarding the brown seaweed *Cystoseira abies-marina,* were presented in the 49th West European Fish Technologists Association (WEFTA) Conference that was held in Tórshavn, Faroe Islands between 15th and 17th October 2019. The poster is illustrated in **Figure 16**.

Lipid Composition of the brown seaweed *Cystoseira abies-marina* and the red seaweed *Asparagopsis taxiformis*

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Introduction

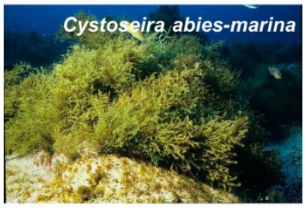
- Seaweeds (SW) an important part of marine food web, are still an underexploited resource and represent as food a smaller carbon footprint
- SW have nutritional value ⇒ **health benefits**

This work intend to characterize the lipid composition of a brown, *Cystoseira abies-marina*, and a red, *Asparagopsis taxiformis*, SW species.

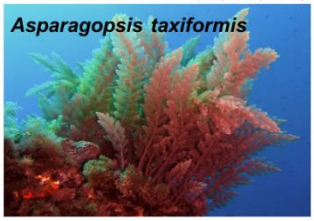
Methods & Results

Samples
SW harvested in the Azores islands (in mid-North Atlantic)

Phaeophyceae (Brown)



Rhodophyta (Red)



Analysis
Total lipid content (liquid-liquid extraction, Folch method), main lipid classes (TLC), and fatty acid (FA) profile (GC-FID) were determined.

Lipids (g/100g dw)

Seaweed	Total lipid
C. abies-marina	0.79±0.13
A. taxiformis	3.40±0.29

Fatty Acids (% of total FA and mg/100g dw)

	C. abies-marina		*A. taxiformis*	
Fatty acid	%	mg/100 g dw	%	mg/100 g dw
14:0	6.8±0.2	37.8±1.4	15.6±1,0	98.6±12.5
16:0	34.3±1.3	189.5±7.3	57.1±2.3	375.5± 56.7
∑SFA	**46.9±0.2**	**259.2±1.2**	**76.2± 3.2**	**501.5± 77.3**
18:1 ω9	18.2±0.2	100.9±1.4	4.0± 0.1	26.3± 2.4
18:1 ω7	0.2±0.0	0.8±0.2	2.1± 0.4	13.5± 1.8
∑MUFA	**21.0±0.1**	**116.3±0.8**	**9.8± 1.5**	**63.8± 5.4**
20:4 ω6	15.2±0.5	84.2±2.8	1.7± 0.4	11.1± 1.7
20:5 ω3	1.9±0.1	10.3±0.5	8.6± 1.7	55.5± 4.9
∑PUFA	**30.2±0.6**	**167.1±3.3**	**10.5± 2.5**	**67.9± 8.4**
ω3	**8.5±0.2**	**46.9±0.9**	**8.6± 1.7**	**55.4± 4.9**
ω6	**21.4±0.5**	**118.5±2.7**	**1.9± 0.9**	**12.4± 3.6**

Lipid Classes (% of total lipid classes)

Seaweed	PL	ST + 1,2 DAG	1,3 DAG	FFA	TAG	Others
C. abies-marina	5.1±1.2	13.6±0.4	5.9±0.2	7.9±0.6	13.2±0.1	54.3±1.8
A. taxiformis	14.0±0.4	1.6±0.83	nd	nd	nd	84.4±1.3

PL- Phospholipids; ST – Sterols; DAG – Diacylglycerols; FFA- Free fatty acids; TAG- Triacylglycerols; nd – not detected

The results showed differences between these SW species. However, lipid levels were low for both species. It was found that each SW species had a particular FA profile. For both SW species, high percentages of palmitic acid (16:0) and low levels of n-3 polyunsaturated FA were registered. A deep characterization of unknown lipid classes will be carried out in future work.

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49TH WEST EUROPEAN FISH TECHNOLOGISTS' ASSOCIATION CONFERENCE, TÓRSHAVN, FAROE ISLANDS, 14 - 18 OCT 2019

Figure 16 - Poster presented in 49th WEFTA conference, held in Faroe Islands between 15th and 17th October 2019.